SCREENING FOR EFFICIENT ARBUSCULAR MYCORRHIZAL (AM) FUNGAL SPECIES AND RHIZOBIAL STRAINS FOR ENHANCING YIELD IN SELECTED LOCAL CULTIVARS OF *VIGNA UNGUICULATA* (L.) WALP (COWPEA)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT for THE DEGREE of

DOCTOR OF PHILOSOPHY

IN THE SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY
GOA UNIVERSITY

By

DHILLAN MHALGO VELIP

GOA UNIVERSITY

TALEIGAO

GOA

MAY 2022

DECLARATION

I, Dhillan Mhalgo Velip hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Taleigao Plateau.
Date : 16-05-2022

Dhillan Mhalgo Velip

CERTIFICATE

I hereby certify that the above Declaration of the candidate, Dhillan Mhalgo Velip is true and the work was carried out under my supervision.

Prof. B. F. Rodrigues
(Research Guide)
School of Biological
Sciences and Biotechnology (Botany)
Goa University

ACKNOWLEDGEMENTS

I am sincerely grateful to all the individuals who have kindly given me advice and support in various ways to the point I have reached for the submission of this thesis. I owe my most sincerely gratitude to my guide, Prof. B. F. Rodrigues, for his invaluable support, guidance, encouragement and patience throughout this research work. I further thank him for all his meticulous suggestions and helpful discussions that guided me in the right direction.

I gratefully acknowledge the support provided by the Department of Science, Technology and Environment (DSTE), Government of Goa, India, under the project entitled "Screening for efficient microbial consortium for yield improvement in *Vigna unguiculata* (L.) Walp for the fellowship awarded during my Ph. D. work.

I would also like to acknowledge the invaluable administrative assistance provided by Prof. Harilal B. Menon, Vice-Chancellor, Goa University, Registrar, and their subordinates during my Ph.D. work.

In particular, I would like to thank my Vice Chancellors' nominee Prof. M. K. Janarthanam, School of Biological Sciences and Biotechnology (SBSB) (Botany), and Prof. S. Ghadi, Vice Dean (Academics), SBSB & Former HoD, Department of Biotechnology, Goa University, for their scientific guidance and encouraging comments throughout my study period. I would also like to thank Prof. Savita Kerkar, Dean, SBSB, Goa University, for allowing me to conduct my research.

I sincerely thank Prof. S. Krishnan, Vice Dean (Research), SBSB & Former HoD, Department of Botany, for providing the facilities for my research work. I am thankful to all my teachers, Prof. P. K. Sharma, Prof. V. U. Kerkar, Dr. N. M. Kamat, Dr. R. Bhandari, and Dr. S. Jalmi, SBSB (Botany), for their valuable help. My special thanks to Prof. D. J. Bhat, former HoD, Department of Botany, Goa University for his support and encouragement.

My appreciation goes out to the non-teaching staff of SBSB (Botany Discipline), Goa University, especially Mr. Vasudev Gaonkar, Mr. Samrat Gaonkar, Mr. Dilip Agapurkar, Mrs. Nutan Achari, Ms. Teja Naik and Ms. Siddhi Naik for their constant help and cooperation.

I owe a great deal of appreciation and gratitude to Dr. Gopal R. Mahajan, Scientist (Soil Sciences) Natural Resource Management Section, ICAR-CCARI, Ela, Old Goa, for soil analyses and the Director, ICAR-Central Coastal Agricultural Research Institute, Ela, Old Goa for extending help through these facilities. I wish to extend my special thanks to ICAR-CCARI Scientist Dr. R. Ramesh for providing bacterial culture (PGPR) for my research study. I am deeply grateful to Dr. Manohara K. K. for providing seed materials for my research and for his valuable assistance at every stage of my Ph.D. work. I would also like to thank Dr. V. Arunachalam, and Dr. A. R. Desai for their support and encouragement. I would also like to thank Prof. Chandrabhas Narayana, Director, Rajiv Gandhi Centre for Biotechnology, for assisting with DNA sequencing of bacterial cultures at their institute. I am deeply grateful to Prof. C. Rivonker, former HoD of the School of Earth, Ocean and Atmospheric Sciences, Goa University, for allowing me to carry out my soil analysis in their department.

I am indebted to Dr. Pradip Sarmokadam, Member Secretary, Goa State Biodiversity Board, for his appreciable help and support in my Ph.D. work. My sincere gratitude to Dr. Christabelle E. G. Fernandes for her encouragement, support, and valuable assistance throughout my Ph.D. work. I am deeply grateful to Prof. Mehtab Bukhari for her constant encouragement, invaluable advice and help for supporting me since since my undergraduate.

I extend my special thanks to Dr. Kim Rodrigues and Late Dr. Andy Willis for their stimulating discussions and constructive advice. I have really appreciated the wonderful moments to all the members of the 'Mycorrhiza lab' for all those useful inputs, healthy discussions, friendship, and mutual help. I take this opportunity to express my deepest gratitude to my lab-mates, Ms. Tanvi Prabhu, Dr. Sankrita Fal Desai, Ms. Apurva Sawant, Ms. Amisha Shirodker, Mr. Ratish Velip and Mr. Vinayak Khanolkar for their support.

I wish to extend my special thanks to Dr. Wendy Martins, Dr. Ranjita Sawaikar, Dr. Ashish Prabhugaonkar, Dr. James D' Souza, Dr. Anup Deshpande, Dr. Ravikiran Pagare, Mrs. Mithila Bhat and Mrs. Pallavi Konge Ranadive for their continued help and assistance.

I am deeply indebted to Dr. Puja Gawas for constant encouragement, valuable suggestions, and support during my research work. My special thanks to Dr. Suraksha Pednekar and Dr. Pratibha Jalmi for invaluable help throughout my Ph.D. study. I thank all the research students

(past and present) at the SBSB (Botany Discipline), Goa University, for their help. I would also like to thank Ms. Shravani Korgaonker, Ms. Shristi Chahal and Ms. Annie Nadar for their kind assistance during the course study.

I wish to extend my special thanks to Mr. Santoba Rao Desai, Mr. Pritam Naik, and Mr. Pele Travasso for their immense support during the course study. I place on record my special thanks to my friends, Dr. Rajath Chitari and Dr. Kiran Gaude, for their kind assistance. I am deeply thankful to Ms. Kalpana Velip, Ms. Deepali Velip, Mrs. Shaila Velip, and Mrs. Ratan Velip for their ceaseless help during the research. I wish to extend my special thanks to Mr. Devanand Kavlekar for his kind support and help during the course study.

I want to extend my special thanks to Adv. Bruno Rodrigues for providing a paddy field area for conducting experimental work throughout my research.

I take this opportunity to express my deepest gratitude to my close friend Mr. Dilraj Fal Dessai, for his unrelenting support and help during my research work. I am also very much thankful to my friend Mr. Akshay Parab, Mr. Gautam Lotlikar, Mr. Vaman Gaonkar, Mr. Uday Gaonkar and Mr. Suraj Kalangutkar for their help, moral support, and strong encouragement during the course study. My special thanks to Mr. Nitin Prabhu, Mrs. Nabha Prabhu and Ms. Mauli Prabhu for their constant moral support during the study.

I reserve my most profound gratitude to my beloved parents for their unconditional love and belief in my abilities towards the completion of this course. I am extremely grateful for constant support and trust from my family. Above all, I would like to thank the almighty God for granting me countless blessings, knowledge, and opportunity to complete my goal. Lastly, I thank all who helped me directly or indirectly in their respective capacities to complete this work.

Dedicated

To

My

Loving

Parents

TABLE OF CONTENT

TABLE OF CONTENT

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF PLATES

CHAPTER 1: INTRODUCTION

1.1: Cowpea introduction

Vigna unguiculata (L.) Walp. (cowpea) (2n=2x=22) is a self-pollinating climbing herbaceous annual legume belonging to the family Fabaceae and is grown for its edible greens, seeds, and pods. The cowpea has several common names and is often referred to as niebe, wake, ewa in West Africa, frijol caupí and feijão-caupí in Brazil, southern peas, blackeyed peas, field peas, pinkeyes, and crowders in the United States. This grain legume is locally known as '*alsando*' in Goa.

The legume is well adapted to the warm tropical environment and grows well in sandy, poor, acidic soils and regions having rainfall between 350 mm and 900 mm. It produces high biomass and has a high N fixation capability. It is tolerant to heat and drought stress and grows optimally at about 28^0C. This characteristic has led the legume to occur in the arid, semi-arid, tropical parts of the World (Singh *et al.*, 2002). As per Pasquet (2000) cowpeas were classified into cultivar groups such as *biflora*, *sesquipedalis*, *melanophthalmus*, *unguiculata* and *textilis.*

Cowpea is thought to have originated in Africa and has spread throughout the world through various trade routes. Its adaption to the typical warm-season tropics has resulted in it being one of the most multifunctional pulse food crops in the arid, semi-arid, tropical parts of Asia, South Europe, Oceania, Africa, South and Central America, and the United States (Singh *et al.*, 2002). It was believed that despite considerable morphological diversity, a single domestication event may have given rise to all cultivated varieties resulting in limited genetic diversity of the cowpeas (Fang *et al.*, 2007). The International Institute of Tropical Agriculture (IITA) has the world's largest and most diverse cowpea germplasm collection. Over 15,000 unique accessions from 88 countries have nearly 70% representing African cultivars and almost half the global diversity (Boukar *et al.*, 2012).

1.2: Economic and nutritional importance of cowpea

Cowpea is an economically significant, nutritionally important, and agriculturally essential legume in developing countries and the livelihoods of millions of poor people depend on it. (Timko *et al.*, 2008). The legumes above-ground biomass except pods may be harvested

for fodder, and hence it is a dependable revenue-generating commodity for farmers, grain traders, and smallholder farmers (Walker, 2016). In India, cowpea assumes a special significance in the food and nutritional security of the people as it is recognized as a readily available and affordable source of proteins. The two major cowpea species grown in India include, *V. sesquipedalis* (L.) Verde. (Yardlong beans) and *V. unguiculata* (L.) Walp. That have a wide variation in plant type, seed, and hilum colour.

Cowpea acts as a valuable source of dietary protein and carbohydrates, thus providing feed, forage, hay, and silage for livestock (Alemu *et al*., 2016; Belay *et al*., 2017). As a vegetable the young leaves and tender, immature pods of cowpea are used (Nielsen *et al.*, 2004) or dry beans as a pulse or a snack. The legumes possess a definitive source of nutrients with appreciable vitamins and minerals and many bioactive compounds having beneficial metabolic and physiological effects on the body primarily when used frequently in the diet. It consists of several nutraceuticals properties such as dietary fibre, polyunsaturated fatty acids, antioxidants, and polyphenols (Trinidad *et al*,. 2010; Shetty *et al.,* 2013; Baptista *et al.*, 2016), numerous bioactive compounds including enzyme inhibitors, lectins, phytates, oligosaccharides, saponins, and phenolic compounds (Bouchenak and Lamri-Senhadji, 2013).

The vegetable is an essential source of protein, vitamins, and minerals such as potassium (K), phosphorus (P), magnesium (Mg), calcium (Ca), selenium (Se), iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn) (Singh, 2006). The cowpea grain contains an average of 23-25% protein and 50-67% starch, and some phenolic compounds (Belane and Dakora, 2009). Several studies have shown that legumes may prevent breast cancer (Adebamowo *et al*,. 2005) and lower the risk of cardiometabolic disorders (Bouchenak and Lamri-Senhadji, 2013). Besides, the phenolic compounds in the seeds have been shown to have anti-viral, anti-inflammatory, anti-bacterial, anti-allergenic activity, and anti-diabetic activity (Siddhuraju and Becker, 2007).

In the agricultural system, cowpea is also known as the 'hungry-season crop' as it is the first crop to be harvested before the cereal crops (Gomez, 2004). Additionally, the legume is also known to maintain the productivity of soils as a cover crop, green manure crop, N-fixing crop, prevents soil erosion, and have weed-suppressing ability (Alemu *et al*., 2016; Belay *et al*., 2017). The ability of cowpea to grow fast and bushy provides the capacity to

cover the ground quickly, suppressing the weeds and protecting the soil against erosion. In addition, after the crop is harvested, the root residue decays and contributes to the organic matter and associated nutrients within the soil.

Cowpea is considered an important rotational crop due to its ability to improve the soil N levels with no chemical fertilizers. This is due to its unique ability to fix atmospheric N through symbiotic interactions with microbial symbionts such as *Rhizobium, Bradyrhizobium*, and AM Fungi (Sarr *et al.*, 2015). As a result, the soil's enhanced N and P fertility benefits other cereals crops (Hall, 2012). The cowpea legume is grown as a mono-crop or intercropped effectively with sorghum, maize, cassava, millet, and cotton (Karanja, 2014). It is considered a key player in sustainable farming in semi-arid lands with sandy loam texture, moderate to low natural fertility, and low external inputs. Here, the intercropping of grain crops (sorghum and millet) with cowpea is common. The cowpea compensates for the loss of N absorbed by cereals, thus positively impacting soil properties.

1.3: Biological Nitrogen Fixation (BNF)

Although about 78% of the atmospheric air is N, most living organisms cannot use the gaseous form and must be fixed. Moreover, N is a vital element for plant growth and development and is required to synthesize macromolecules such as amino acids, nucleic acids, and chlorophyll. Its deficiency in the soil adversely affects plant growth (Uchida, 2000). In agriculture, the lack of N translates into a lower yield. Thus, fertilization with N products is practiced to increase the output (Xu *et al.*, 2012). There has been a substantial usage of N fertilizer in both developed and developing countries (Peoples *et al*., 1995). However, the use of N fertilizers have a negative and unpredictable impact on the environment due to leaching, volatilization, and denitrification, which has contaminated the soil, water, and other natural reservoirs, thereby affecting the health of humans and animals. Besides, application of organic fertilizers have also increased the production cost of crops.

The increasing global concern regarding the demand for the production of crops required for the growing human population, and at the same time, concern for the degradation of the environment caused by adverse effects of fertilizer usage is fortifying the importance of intensifying plant production sustainably. Currently, the use of microorganisms capable of

fixing atmospheric N through BNF is of immense practical importance because it makes it possible to limit the usage of chemical fertilizers. Its use has been exploited extensively in agricultural practice, especially in growing legumes (Krasova-Wade *et al*., 2006). It is believed to be an efficient source of N (Peoples *et al*., 2009), wherein N's total annual terrestrial inputs from BNF range from 139-175 million tonnes of N (Verma *et al*., 2020). In agricultural production, the legume-*Rhizobium* symbiosis contributes at least 50% of the 175 million tons of N per year (Mabrouk and Belhadj, 2012).

The formation of nodules on roots and stems by rhizobia which is a characteristic feature of the symbiotic association between rhizobia and leguminous plants (Graham, 2008). The bacteria encounter optimal conditions for BNF by forming nodules. The nodule formation is a highly regulated process dependent on the exchange of specific signaling molecules between the micro- and the macro-symbiont (Oldroyd *et al*., 2011). The nitrogenase enzyme in the nodules reduces N_2 into NH_3 via the GS/GOGAT pathway, leading to the exchange of nitrogenous solutes with the host plant for recently-formed photosynthate.

1.4: Rhizobia and cowpea

It is known that the cowpea yield is enhanced based on the contribution of effective rhizobia and a proper management system. The variation in N fixation differs environmentally due to soil temperature, moisture, rhizosphere mineral nutrition, different cowpea genotype, management of agro-ecosystem, abundance, and efficacy of rhizobia in the soil (Dakora *et al.*, 2015; Dwivedi *et al.*, 2015). Information on the diversity of cowpea nodulating rhizobia in most parts of the agricultural system is limited. However, it is believed that large diversity of rhizobia groups exists in soils. In general, cowpea is nodulated by slow-growing rhizobia belonging to the genus *Bradyrhizobium*. Fast-growing rhizobia have also been reported to nodulate cowpea (Chidebe *et al*., 2018; Silva *et al*., 2012).

Studies in African countries have shown that genus *Bradyrhizobium* (Pule-Meulenberg *et al*., 2010; Grönemeyer *et al*., 2014) and genus *Rhizobium* (Mpepereki *et al*., 1996) caused nodulation in cowpea. However, various other countries have reported cowpea nodulation by *Rhizobium*, *Sinorhizobium*, *Ralstonia*, *Achromobacter*, and *Microvirga* (Martins *et al*., 1997; Zhang *et al*., 2008; Leite *et al*., 2009; Sarr *et al*., 2011; Guimaraes *et al*., 2012; Radl *et al*., 2014).

In India, rhizobia related to bradyrhizobial species isolated from *Glycine max* (as a new bio-variety) could nodulate soybean and *Vigna* species as a new lineage within the *Bradyrhizobium* genus. However, the soybean varieties grown by farmers in Africa generally nodulate with indigenous cowpea-type *Bradyrhizobium* (Jaiswal *et al*., 2016; Chibeba *et al*., 2017). Plant growth and grain yield of these legumes increase with enhanced N fixation.

Several environmental factors such as pH, clay, and organic matter affect the diversity of soil, growth, and rhizobia activity. These features can shape soil and plant-associated habitats, modifying the composition and actions of their microbial communities. Further, soil pH has been found to influence bradyrhizobial diversity, while clay and organic matter contents have little influence on bacterial diversity (Wang *et al*., 2016). This may be because the pH affects the bioavailability of mineral nutrients in soils.

1.5: Arbuscular Mycorrhizal (AM) fungi

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi that form a symbiotic association with plant roots (Smith and Read, 2008), belonging to the phylum Glomeromycota. The phylum is an ancient form of symbiosis in plants and is considered a monophyletic group. Soil microbes are of great significance, as they are responsible for most biological transformations, including nutrient recycling, thereby facilitating the subsequent establishment of plant communities (Schulz *et al*., 2013). Schüßler and Walker (2010) propounded a single class of Glomeromycetes with four orders, 11 families, and 18 genera. About 288 taxonomically described species are currently included in this group (Öpik and Davison, 2016). The most recent classification of Glomeromycota is based on a consensus of regions spanning rRNA genes: 18S (SSU), ITS1-5.8S-ITS2 (ITS), and 28S (LSU) **(Fig. 1.1)**. Redecker *et al*., (2013) discussed the phylogenetic reconstruction underlying this classification.

The AM fungi are complex microsymbionts globally distributed and considerably impact the biotic, edaphic, and spatio-temporal affecting all terrestrial niches (Willis *et al*., 2013). The primary function of AM fungi is to contribute to the plant's nutrition, especially in nutrient-depleted hostile environments, in the form of P, which is often a limiting resource (Bolan, 1991) and micronutrients (Clark and Zeto, 2000). Besides, AM fungi play a role in

reducing the uptake of phytotoxic heavy metals (Göhre and Paszkowski, 2006), controlling root invasion (Newsham *et al*., 2009), reducing water stress to host plants (Auge, 2001), aggregating of soil particles through Glomalin (Rillig and Mummey, 2006), reduces insect herbivory (Bennett *et al*., 2009), increases insect pollination, insect density of trophic food web (Gange and Smith, 2005; Hoffmann *et al*., 2011), increases soil community population and organization in structure patterns (van der Heijden *et al*., 2008; Rillig *et al*,. 2006). AM fungi produce arbuscules, which assist in the exchange of inorganic minerals and the compounds of C and P (Li *et al*., 2016; Prasad *et al*., 2017), suggesting that these fungi play a significant role in soil N and C cycles (Jones *et al*., 2009) thereby making a considerable contribution to terrestrial ecosystem C sinks (Wright and Upadhyaya, 1998).

The AM fungal growth and development are dynamic and rapid. Roots exudate encourages germ-tube growth towards the roots at the pre-symbiotic stage (Sbrana and Giovannetti, 2005). The extent of AM fungal root colonization varies based on the host plant species (Klironoms, 2003), host phenology, soil biota interactions (Dauber *et al.,* 2008), and C allocation (Muthukumar and Udaiyan, 2000). The number of arbuscules occupying the root length varies within fungal species (Fitter, 2006), season, soil hydrology (Schreiner *et al.,* 2007), and soil temperature (Smith and Read, 2008a). AM species diversity and abundance are based on sustainable soil fertility and organic agriculture (Willis *et al.*, 2013). These fungi play an essential role in sustainable agricultural practice and ecological restoration. AM fungal root colonization in the host changes significantly over time, competing with each other, causing variations at the taxonomic level (Hart and Reader, 2002).

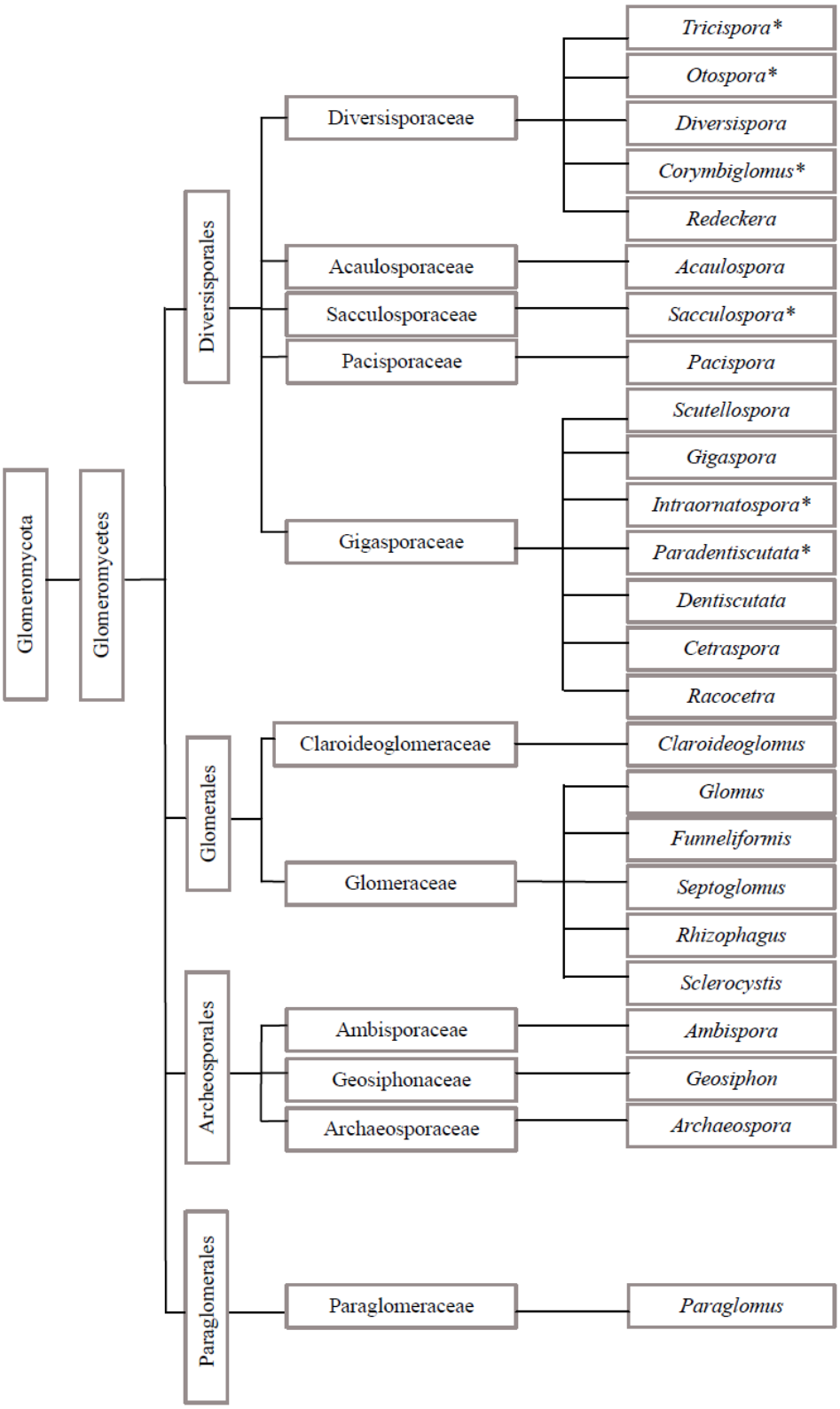


Fig. 1.1: Consensus classification of AM fungi by Redecker *et al.*, 2013. (*designates the uncertain position of genera).

Applications of agricultural chemical fertilizers, fungicides, and pesticides have been shown to have both negative and positive effects on AM fungal population (Willis *et al.*, 2013). AM fungal extra-radical hyphae in leguminous plants contribute indirectly to N only in reduced P conditions, supplying essential P and micronutrients to N fixing organisms (Smith and Read, 2008). The potential use of AM fungi and plant growth-promoting rhizobacteria in agriculture and horticulture is slowly being recognized (Azcón, 2000; Lucy *et al.*, 2004). It is also highlighted that N fixation and P solubilization in the rhizosphere and phytohormones production can directly enhance plant growth. Therefore, AM fungi offer great potential for sustainable agriculture as they would also help reduce the excessive use of P fertilizers. This is possible because of their high metabolic rate and strategically diffused distribution in the upper layers of the soil. Therefore amendment of soil with biofertilizers such as vermicompost, farmyard manure, and AM fungi is essential to overcome nutrient deficiency, neutralize acidity, and enhance the soil's microbial activity to establish plant growth.

1.6: Cultivation practices and economic importance of cowpea in Goa

Agriculture is the third leading source of livelihood in the state of Goa, providing livelihood to about ten percent of the population. Goa is the smallest state of India, having $15^0 48' 00''$ N and $14^0 53' 54''$ N Latitude and $74^0 20' 13''$ E and $73^0 40' 33''$ E Longitude with a total geographical area of 3702 sq. km (Gune, 1979). The State of Goa, which lies on the West Coast of India, has favourable factors for increasing the production of many agriculture and horticulture crops. The state receives high rainfall of about 2800-3000 mm with fertile soils and undulating terrain. Further, the region's warm, humid tropical climate favours flora and fauna for growth and development. It is one of the wealthiest ecosystems (ICAR Vision 2030, 2011). The region's low-lying areas are dominated by cropping systems, i.e. rice and rice-based systems, while the uplands are dominated by cashew and coconut plantations. Further, the rice-pulse, rice-groundnut, and rice-vegetables methods are practised in Goa. However, the productivity levels of vegetables and pulse crops are low compared to national data. The crop's cost and yields can be improved by introducing new agricultural and technological inputs.

Cowpea, is a major pulse crop in the West Coast region. The cowpea produces long bold, fleshy pods, with bold seeded variety and vine producing higher biomass. ICAR-CCARI for Goa has identified three local cowpea selections, *viz.*, Alsondo-l, Goa Nadora (Nadora

Bardez-4), and Dhulape Utorda-3 (Goa Cowpea-3), each with specific advantages. Being tolerant to drought, it is grown extensively as a fallow rice crop under residual moisture conditions. Cowpea is usually grown in sandy loam, sandy, alluvial and lateritic soil with good water holding capacity. It requires a warm and humid climate with a temperature between 29-35^{0}C. Excellent response to its cultivation is observed in soils having organic carbon (>0.6 %). It is cultivated in Kharif (June to September), primarily for green leafy vegetables in lateritic uplands (morod) and as an inter-crop during rabi season. The sowing time is vital and is carried out from the second fortnight in November to the first fortnight of December. The crop is exposed to moisture stress if sowed beyond January during the rabi season, leading to delayed seedling emergence and seedling vigour. Further, each phase of cowpea development attracts several insect pests and diseases. The major insect pest includes Aphid (*Aphis craccivora*), Gram pod borer (*Helicoverpa armigera*), Spotted pod borer (*Maruca testulalis*), Leafhopper (*Empoasca kerri*), Pod bugs (*Riptortus pedestris*), and Whitefly (*Bemisia tabaci*). The acute diseases of cowpea include root rot, damping-off, wilt, and leaf mosaic.

Cowpea is known to fix 45-150 kg atmospheric N per hectare. When grown as an intercrop, it is known to suppress weeds (20-45%). It demands no irrigation, but many farmers practice irrigation, which improves soil biotic life while enriching soil fertility. About 35-40 kg of seeds are required to plant an area of one hectare with required spacing (45cm for Alsondo-1, 60 cm for Goa Nadora and Goa Cowpea-3) in straight lines, grown at a depth of 5cm in the moist zone with a recommended fertilizer application of 10-25 kg N, 50-60 kg P_2O_5 and 25-30 kg K_2O. The harvest comes up quickly without much management as the crop matures in 100-110 days, showing signs of brown discolouration and gradual drying. Manohara *et al.,* (2021) reported that the yield of local type varieties is generally low, with an average seed yield of 0.8-1.0 t ha^{-1}. The pods are sun-dried for three to four days till they become brittle, and grains are separated by trampling the pods under feet or using wooden sticks. Although a few high-yielding types of small-seeded cowpea are produced, they are not popular among farmers. However, the Goan cowpea is popular and used in many culinary preparations. The cowpea draws premium prices due to its bold size, soft texture, better cooking quality, and exquisite taste (Manohara *et al.,* 2021).

The importance of legumes in the human diet is expected to increase to meet the demand for protein and other nutrients in the growing world population. The selected local

cultivars should be studied in terms of cultivation practices in the region. A literature review indicates no studies have been reported on *alsando* related to yield using bio-fertilizers. Hence, this work is attempted to bridge the gap and encourage the use of potential local varieties. However, to further popularize these varieties among the farmers, there is need to improve their yield. The present study was initiated with the following aims and objectives.

1. A detailed survey of *V. unguiculata* growing areas and identification of various cultivars from Goa.
2. Physico-chemical characteristic of *V. unguiculata* grown soils.
3. Study on the AM fungal diversity in different cultivars of *V. unguiculata*.
4. Isolation and molecular identification of the rhizobial strains associated with *V. unguiculata*.
5. Standardization of mass multiplication of AM species using pot cultures and *in-vitro* techniques.
6. Formulation of the standard carrier for inoculum (both AM and *Bradyrhizobium*).
7. Evaluation of promising AM fungi and rhizobial strains for increased yield in cowpea.

CHAPTER 2: REVIEW OF LITERATURE

2.1: *Vigna unguiculata* (cowpea)

Cowpea is a drought-tolerant forage that replenishes low fertility soils, tolerates shade, and is a cover crop to prevent soil erosion (Dugje *et al*., 2009). Besides, it serves as human food, fodder for livestock, and green manure (Singh *et al*., 2003) and contains numerous minerals and vitamins. The immature seeds can be consumed, while the young pods and leaves are used as edible vegetables and livestock fodder (Timko and Singh, 2008). Okonya and Maass (2014) reported that in more than 18 African countries, tender leaves are consumed as leafy vegetables.

2.2: Challenges facing cowpea production

Cowpea is mainly grown by smallholder farmers. These farmers play a crucial role in domestic nutrition and food security while significantly contributing to global food production (Dioula *et al*., 2013). The dried cowpea production is estimated at 6.2 million tons grown on 14.5 million ha of land across the globe (Kebede *et al*., 2020), with 96% originating from Africa. The yearly global production increases with the average yield per hectare increase with a subsequent increase in the revenue. It is observed that most smallholder farmers face low soil fertility management and cannot use sufficient fertilizers to replenish the soil.

The cowpea crop is affected by several insects and pests (Gungula and Garjila, 2005). Oyewale and Bamaiyi (2013) reported flowering and post-flowering insect pests that include leaf miners, whiteflies (*Bemisia tabaci*), leafhoppers (*Empoasca* sp.), mites (*Tetranychus* spp.), thrips *Megalurothrips sjostedti*, *Ootheca* sp., *Clavigralla* sp., *Maruca* sp. and aphids (*Aphis craccivora*). This generally results in low yield or total yield loss and crop failure. These pests severely attack the crop at different growth stages. The pod-sucking bugs *viz., Clavigralla tomentosicollis, Riptortus dentipes,* and *Anoplocnemis curvipes* are the major post-flowering pests that significantly reduce yield (Oyewale and Bamaiyi, 2013). Some farmers use insecticide sprays, while others use tolerant varieties locally available. Disease and pest resistance genes have been incorporated into cowpea (Dzemo *et al*., 2010). Use of eco-friendly and sustainable agricultural practices to reduce the increasing cost of yield through chemical fertilizers and pesticides has been suggested

for the cowpea crop. It is indicated that smallholder farmers can practice environmentally sustainable measures such as bio-fertilizers (using AM fungal inocula) and bio-pesticides containing microbes that enhance plant growth and increase crop productivity.

2.3: The arbuscular mycorrhizal (AM) fungi

2.3.1: Ecology and distribution of AM fungi

The term 'Mycorrhiza' was coined by Frank, B. (1885) (Mycos= fungus, rhiza= roots) to describe the symbiosis between plant roots and a soil fungus (Wang and Qiu, 2006). According to Brundrett and Tedersoo (2018), nearly 72% of vascular plants are associated with AM fungi, around 10% have orchid mycorrhizae, and 2% are ectomycorrhizal, while about 1.5% have an ericoid association. Among the mycorrhizae, the AM fungi are the most prevalent type found in about 80% of the terrestrial plant families (Barrow *et al*., 2008).

They are beneficial to soil biota and have existed for more than 400 million years (Bainard *et al*., 2011). They are found in diverse landmasses such as arid/semi-arid grasslands, calcareous grasslands, tropical rain forests, and temperate forests. Smith and Read (1997) reported widespread distribution of AM fungal symbiosis in angiosperms compared to pteridophytes and in some gymnosperms and gametophytes of lower plants such as lycopods and mosses. Moreover, the AM fungal diversity varies depending upon soil type, regions, indigenous crops, medicinal plants, dunes vegetation, and other plant species (Khade and Rodrigues, 2009; Radhika and Rodrigues, 2010; Dessai and Rodrigues, 2012).

Manjarrez *et al*., (2009) reported that AM fungi colonization varies based on the genetic variability of host species. An *et al*., (2010) reported genetic variation in 255 maize genotypes from different locations, including inbred lines, hybrids, and landraces. The host plant, its fungal partner, and its ecological habitat (soil) form a significant association in an ecosystem. This extensive fungal hyphal network captures complex compounds, and other inorganic nutrients, especially P from the soil and hexose sugars, namely carbon (C), and the nutrients flow to the fungus (van der Heijden *et al*., 1998). The AM fungi form arbuscules which are highly branched intra-cellular tree-shaped structures (Koide and Mosse, 2004), being the sites for nutrient exchange between the two symbiotic partners

(Smith *et al*., 2011). Thus, these structures are formed when nutritional transfer between the phytobiont and the mycobiont occurs (Peterson *et al*., 2008).

The plant diversity, plant community structure, ecosystem productivity, and the ecosystem's function and ability to maintain itself have a significant role in the composition and diversity of AM fungi. Besides, studies have shown that AM fungi respond differently to soil fertility, pH, organic carbon, moisture, P, and other cations (Smith and Read, 1997; Koide and Mosse, 2004). AM fungi improve the soil's physical quality, protect the host against soil-borne pathogens, stimulate plant growth, and are connected to the topsoil layer through extensive hyphal networks (Avio *et al*., 2006). These fungi act as a primary component of the microbial population and improve nutrient supply and productivity (Johansson *et al*., 2004). Azcón and Barea (2010) have also reported the significant role of AM fungi in nutrient mobilization, carbon cycling, different environmental stresses, heavy metal toxicity, soil salinity, heat stress, drought, plant pathogen, and soil acidification. In AM fungal symbiosis, the fungus plays an essential role in plant roots hair, increasing the lifespan of absorbing roots due to its extensive hyphae extensions, extending to cover larger soil areas (Muchovej, 2004). Further, losses through leaching or reactions with soil colloids are reduced, thus enhancing plants' retention and better utilization of soluble nutrients (Selvaraj and Chellappan, 2006).

Rachel *et al*., (1989) carried out studies on the distribution of AM fungi in the Indian semiarid region. Khade and Rodrigues (2009) studied the occurrence of AM fungi in the agro-ecosystem. Change in the AM fungal population has also been observed in agricultural and vegetable crops (Dessai and Rodrigues, 2012). Studies on AM fungal diversity based on the geographical distance, rainfall, and different seasons have been carried out earlier (Oliveira and Olivera, 2005; Radhika and Rodrigues, 2010).

2.3.2: The influence of AM fungi on the agricultural ecosystem

Phosphorus (P) and N are the primary nutrients needed for plants' growth, obtained through supplied fertilizers or manures. In an agricultural system, AM fungal community characteristics are affected due to the application of chemical fertilizers, pesticides, and fungicides. Synthetic fertilizers and fungicides are known to drastically decrease the AM colonization levels in the farming system. The fertilizer application is known to reduce the mycorrhizal diversity and abundance due to an increase in available P. Hijri *et al*., (2006)

have reported that the mycorrhizal colonization inhibits residual P levels, even after conversion to organic systems. Abd-Alla *et al.*, (2000) have shown that pesticides significantly inhibit AM colonization and spore production in legume crops.

2.3.3: AM inoculum production

The AM fungal association plays a significant role in the uptake of crop nutrition, and therefore, there is increased growth and yield (Koide *et al*., 2000). As a result, desirable traits of AM symbiosis with various cultivars have been widely selected in agriculture. However, there is a constant need to study AM fungi's life cycle and develop contamination-free large-scale inoculum production. Various techniques have been developed for the large-scale production of AM inocula. The usual source of AM inoculum is root segments and spores isolated from open-pot culture (Gilmore, 1968). However, this technique has disadvantages as it occupies an ample space in production and is prone to contamination (Ames and Linderman, 1978).

Fortin *et al.,* (2002) studied the life cycle of AM fungi *in situ* by using the root organ culture (ROC) technique. This non-destructive technique helps learn the fungal life cycle and various aspects of AM symbiosis. Studies have also shown tremendous improvements using Ri T-DNA transformed roots to induce sporulation, manipulation in culture media to transport mineral nutrients to roots, microbial-free fungal mycelium and spores for molecular analysis (de Boulois *et al*., 2005). The successful cultivation of AM fungi associated with the Ri T-DNA transformed roots has provided many insights into the symbiosis. This technique can be exploited for large-scale inoculum production. In this technique, the host plant is replaced by Ri T-DNA transformed roots and the fungus can colonize and sporulate. Besides, spores developed are morphologically and structurally similar to those produced in pot cultures. Also, the fungus can retain the ability to produce propagules that can colonize and initiate new mycorrhizal symbiosis, indicating that the fungus can complete its life cycle. The ROC has been used to culture many species and strains of AM fungi.

In monoxenic culture, extra-radical spore, intra-radical root, or isolated vesicle are used to initiate culture. *Gigaspora* and *Scutellospora* species do not produce vesicles; hence, spores are used for germination. Further, ROC using carrot roots has been used for several *Glomus* species (Diop, 1995). *Rhizoglomus intraradices* is the most abundant and

frequently used species for AM fungi *in vitro* culture. The separated intra-radical vesicles from roots and spores represent a valuable source of inoculum (Plenchette and Strullu, 2003). Declerck and Angelo-van Coppenolle (2000) demonstrated that pathogen-free and high-quality spores of *Rh. intraradices* could be produced in alginate beds using the cryopreservation technique. Rodrigues and Rodrigues (2015, 2017) showed that AM fungi *viz., Rhizoglomus intraradices* and *Funneliformis mosseae* were successfully cultivated to produce mass inoculum using the ROC technique. Adholeya *et al*., (2005) reported large-scale AM fungi and root organ culture using a small container. A container-based hydroponic culture system patented by Wang (2004) was also used where the root organs and AM fungus were periodically exposed to a liquid culture medium. Petri plate containing ROC was also used to initiate fungal proliferation in a separate compartment filled with sterile expanded clay balls (Gadkar *et al*., 2006). Declerck *et al*., (2005) highlighted that these revolutionary research techniques could help overcome AM fungi culturing in the soil.

2.3.4: Crop nutrition: Interaction of AM fungi with cultivars

AM fungi are crucial for maintaining plant nutrition in organic and low-input farming systems (Cavagnaro *et al*., 2012). AM fungi produce significant biomass in agricultural soils (Rillig *et al*., 1999). Further, AM colonization increases the absorptive surface area and life span and exposes roots to the soil. An extensive branched AM mycelial network is spread within the soil to take up nutrients to which most of the plant's ecosystem is connected (Avio *et al*., 2006). Various studies have been carried out on how plant species and cultivar types respond to AM fungi. The improved cultivar was less responsive to AM fungi than landrace in wheat (Tawaraya, 2003) and barley (Zhu *et al*., 2003). However, there are reports of modern cultivars demonstrating a high level of AM colonization (Steinkellner *et al*., 2012). Hildermann *et al*., (2010) recorded increased P uptake efficiency in modern cultivars. Thus, there is still ambiguity about whether the age or crop improvement of the cultivars reduces AM fungal response.

2.4: Symbiotic benefits of AM fungi

Root colonization by AM fungi significantly improves the uptake of immobile nutrients such as P and Zn and increases Ca, N, and K (Balakrishnan and Subramanian, 2012). Mycorrhizal fungi contribute immensely to the soil structure on an ecosystem scale. The symbiotic benefits of AM fungi include resistance to drought and heavy metal toxicity, and

tolerance to root pathogens in plants. Besides, it brings about an increase and improvement in soil structure and aggregation. Studies have reported higher uptake of nutrients in AM plants than in non-AM plants (Sharma *et al*., 2013).

2.4.1: Plant pathogens

It is well known that one of the symbiotic benefits of AM fungi to plants is its soil-borne pathogen control. AM fungal colonization in plant roots increases tolerance to pathogens, thereby acting as a biocontrol agent. Gosling *et al*., (2006) observed that AM fungi occupied the root cell and excluded the pathogen. Further, Sylvia *et al*., (2001) reported that AM fungi colonize the roots faster than the pathogen attack. AM fungi increase the uptake of nutrients and enhance plant vigour and resistance against root pathogens and infections (Begum *et al*., 2019).

Further, due to the colonization, anatomical and microbial changes occur in the mycorrhizosphere, thereby protecting against plant pathogens (Jung *et al*., 2012). However, the mechanism's effectiveness against the pathogen depends upon soil and environmental conditions. Baslam *et al.,* (2011) suggested that AM colonization and the release of phenolic compounds inhibit the growth of the pathogen.

2.4.2: Osmotic stress alleviation

The AM fungi form an extensive hyphae network that can improve plant growth by suppressing the unfavourable stress effects (Garg and Chandel, 2011). Further, mycorrhization increases salinity tolerance and reduces oxidative damage (Latef and Chaoxing, 2014), and drought, and soil-borne pathogens (Azcón and Barea, 2010). Similarly, Shafi *et al*., (2011) reported that salt stress inhibited photosynthesis and progressive plant growth. Further, plant cells produce organic osmoprotectants to overcome plant salt stress-resistant by adjusting osmosis (Bárzana *et al*., 2015). AM fungal association increases higher gas exchange rate, nutrient stomatal conductance, soil pore penetration, water uptake and transfer to host plant, and shoot water transpiration rate.

2.4.3: Drought tolerance

AM fungi regulate stomata by increasing water potential and hydraulic conductivity, cellular water pressure maintenance, osmotic adjustments, and cell wall elasticity changes (He *et al.,* 2017; Zhu *et al.,* 2012). Amerian *et al.,* (2001) observed that non-mycorrhizal

maize plants had lower leaf water potential than those inoculated with *G. mossae* and *G. intraradices.*

2.4.4: Soil aggregation

AM fungi play an essential role in soil aggregation, glomalin production, and soil organic nutrients transfer (Maun, 2009). In a non-mycorrhizal ecosystem, soil aggregation is critical in the context of disturbed land restoration. It controls erosion, soil carbon storage, or global change (Niklaus *et al*., 2003). AM fungi are especially significant as it is one of the most important biological factors contributing to soil aggregation. Rillig and Mummey (2006) have studied the importance of mycorrhiza-influenced soil aggregation at plant communities and root levels. Bedini *et al*., (2009) reported that soil aggregation stability and mycorrhizal root volume in soil aggregation positively correlate with glomalin. It is demonstrated that AM fungi secrete iron-containing glycoproteinaceous substance glomalin, which is the water-soluble, glue-like proteinaceous, and heat-stable substance that helps aggregate the soil structure (Steinberg and Rillig, 2003). It maintains water-stable aggregation and C dynamics in soil. It is used to determine mycorrhizal growth and soil activity (Lovelock *et al*., 2004). It is a recalcitrant accumulating in soils to several mg per cm (Rillig *et al*., 2001) and is generally used to assess soil C in various land-use types.

2.4.5: Ecological restoration

AM fungi act as biological tools for the restoration of a self-sustaining ecosystem. Mycorrhizal symbiosis is the most significant component in ecosystem restoration and occurs in almost all natural habitats (Khade and Adholeya, 2007; Miransari, 2010). Plants inoculated with AM fungi may be introduced to restore degraded soil health (Jeffries *et al*., 2003). Long-term soil inoculation of indigenous AM fungi significantly improves soil quality parameters than non-inoculated or soils inoculated with single exotic species. According to Voets *et al*., (2008), the movement of C occurs through intra-radical mycelium from one root to another. However, AM fungal lipids and plant sugars are stored in the extra-radical mycelium and are essential for mycelia growth and sporulation (Roth and Paszkowski, 2017).

2.4.6: Uptake of P

Phosphorus (P) is the most limiting nutrient for plants to acquire, which is also critical for plant growth and contributes 0.2% of dry mass (Habibzadeh, 2015). Graham (2000)

reported that in the plant-fungal relationship, the status of P is the main controlling factor that benefits the host plant. An AM fungus spreads extra-radical hyphae and secretes phosphate enzymes for hydrolyzing unavailable P sources from the soils (Carlile *et al*., 2001). A high concentration of available P in the soil resulted in AM fungi failing to colonize the plants (Liu *et al*., 2016). It was also noted that when AM fungal colonization is disrupted, there is a significant reduction in the P uptake and, subsequently, the crop yield (Gosling *et al*., 2006). Studies have reported that AM fungal inoculation enhanced P nutrition in cowpea (Yaseen *et al*., 2011), tomato, maize (Miransari, 2011), and cucumber (Ortas, 2010).

Studies have also shown that the hydrolysis process and absorption of P and other nutrients create a depletion zone around the roots, limiting the P uptake rate (Sylvia *et al*., 2001). Hawkins *et al*., (2000) reported that AM fungi obtained NO_3^- or NH_4^+ through their extra-radical hyphae. However, Fe, Mn, Cu, Zn, Ca, and K uptake depends upon P and N concentration. Liu *et al*., (2000) confirmed that AM fungal uptake of Cu, Zn, Fe, and Mn nutrients in maize crops significantly influences P nutrients from the soil. Further, P bioavailability in sandy and clayey soils affects water retention and protects bacteria against grazing protozoa (van Veen *et al*., 1997).

2.4.7: Uptake of other elements

Crop yield is dependent on the levels of micronutrients (Johnson *et al*., 2005). At higher levels, the amount of micronutrients leads to toxicity, while a deficiency is created by low levels of micronutrients (Ashman and Puri, 2013). AM colonization influenced the uptake of nutrients such as K, Na, Ca, Mg, B, Fe, and Zn (Meding and Zasoski, 2008). However, it is known that AM plants contain some elements in higher quantities than non-AM plants. Schreiner (2007) reported an increase in micronutrient uptake in AM inoculated grapevine. Similarly, in different varieties of tobacco inoculated with AM, enhanced uptake of K was recorded (Janouskova *et al*., 2007).

2.5: AM fungi and legume-rhizobia symbiosis

Studies have reported that an increase in root colonization and AM fungal spore germination rate is due to specific soil bacteria and bacterial communities (Johansson *et al*., 2004). At the mycorrhizosphere, growth-promoting rhizobacteria and N-fixing bacteria interact with AM fungi (Gosling *et al*., 2006). Studies have shown that AM fungi influence

legume-*Rhizobium* symbiosis, wherein distinct mycorrhizae were found in root nodules compared to legume roots (Scheublin *et al*., 2004). The N fixation increased nodule formation due to high P nutrition concentration (Vázquez *et al*., 2002). AM fungi and rhizobial plant symbiotic associations are most important for acquiring nutrients.

Rhizobia are gram-negative, free-living, and N-fixing soil bacteria that form a symbiotic association with legume roots (Mus *et al*., 2016). Rhizobia belong to 13 taxonomically diverse genera and have more than 98 species of α and β-proteobacteria (Lira Junior, 2015). In the cowpea symbiosis association, the cowpea can nodulate various symbiotic bacteria such as slow-growing rhizobial species belonging to the genus *Bradyrhizobium* and some bacteria belonging to genera α and β-proteobacteria (Wade *et al*., 2014; Tampakaki *et al*., 2017). Studies have shown that rhizobia fix N, while AM fungi benefit the host plant for P mobilization from non-labile sources (van der Heijden *et al*., 2006). Therefore, the plant gets N through symbiotic dinitrogen (N_2) fixation environmentally friendly (Azcon-Aguilar and Barea, 2015). Legume nodules can host N-fixing bacteria and AM fungi simultaneously as obligate biotrophs. Azcon and Barea (2010) studied the legume rhizobia and AM fungi symbiosis interaction and reported that several legumes did not form nodules in autoclaved soil unless the plants were mycorrhizal. Shockley *et al*., (2004) observed an increase in N fixation with nodule number and weight and assumed that symbiotic N fixation increases due to increased P supply to the nodules.

2.6: An overview of AM fungi, rhizobia, and cowpea production

Cowpea indigenous rhizobial symbiosis is abundant in many tropical soils (Ampomah *et al*., 2008; Kimiti and Odee, 2010). The survival of rhizobia in the ground depends on symbiotic functioning and an essential adequate availability of nutrients such as P, K, Ca, Mg, S, Zn, and Fe, which internally promote plant growth (Zahran, 1999; O'Hara, 2001). In cowpea, the AM fungi increase the atmospheric N fixation and nodulation potential (Turk *et al*., 2006). The increased P uptake by the plants due to AM fungi improves the susceptibility to phytopathogenic fungi or nematodes and increases N fixation by rhizobia (Selvaraj and Chellappan, 2006).

2.7: Rhizobia as inoculants

Nitrogen fixation is complex in root nodules when legume and rhizobia signals are exchanged (Oldroyd, 2013). The atmospheric N fixes in root nodules through the rhizobia-

nitrogenase enzyme and subsequently benefits from malate and succinate (Prell and Poole, 2006). Similarly, bacterial N fixation (BNF) increases crop yield by efficiently utilizing an adequate and sufficient *Rhizobium* at the rhizosphere colonization area (Laranjo *et al*., 2014). An effective rhizobia inoculation practice has been followed for over 100 years to increase crop yield (Herridge, 2008). The technique of inoculation with rhizobia dramatically influences the increase in crop yield and, at the same time, decreases the use of chemical fertilizers (Gopalakrishnan *et al*., 2015). Similarly, several studies have enhanced cowpea productivity based on isolating strains from indigenous rhizobia (Rufini *et al*., 2014; Marinho *et al*., 2017). Martins *et al*., (2003) isolated BR3262 and BR3267 strains used as inoculants in the semi-arid region of Brazil resulting in increased grain yield.

The increase in BNF and fixed N legume production depends on the efficient and competitive rhizobia strains and may also be dependent on a combination of bacterial strains of a legume cultivar. Studies have also suggested that the selection of efficient strains depends upon the N fixation capacity of different rhizobial strains (Ampomah *et al*., 2008; Vieira *et al*., 2010). Fening and Danso (2002) studied the symbiotic effectiveness of 100 isolates from Ghana, of which 68% were ranked moderately and 26% highly effective. In another study, of the five indigenous cowpeas Bradyrhizobia isolates tested for symbiotic effectiveness and competitiveness, only one strain was competitive for nodule occupancy and was recommended for potential use as an inoculant (Ampomah *et al*., 2008).

2.8: Biological nitrogen fixation (BNF) and N fixation by cowpea

Nitrogen is essential for crops and plays a vital role in biological processes such as photosynthesis, enzymatic functioning, and protein production. The N element is used in organic and inorganic fertilizers in the agricultural system from the atmosphere *via* N fixation. However, to produce inorganic N fertilizers, the Haber-Bosch process uses extensive energy, N from the air, high pressure, and temperature with H_2 as catalysts (Renner *et al*., 2015). Further, BNF is a natural means available to the plants to fix atmospheric N (Wagner, 2011).

However, inorganic fertilizers have limited use by smallholder farmers from developing countries, and hence BNF improves soil N and enhances yield. BNF is limited to

prokaryotes, wherein the reduction of N to ammonia occurs in nitrogenase enzyme. This essential N fixing symbiotic association is found between legume and rhizobia (People *et al*., 2009). Herridge *et al*., (2008) estimated that the amount of N fixation from crops was between 33-43 Tg N yr^{-1} and, globally, it was up to 100-122 Tg N yr^{-1}. Similarly, a recent study using the ^{15}N natural abundance method has shown that cowpea fixes 7-23 kg N yr^{-1} (Naab *et al*., 2009). The selection of high N fixing cowpea genotypes is essential to avoid variation in genotype during BNF. Belane *et al*., (2011) reported that N fixation by 32 genotypes from South Africa ranged between 50-182 kg N ha^{-1} while Pule-Meulenberg *et al*., (2010) observed N fixation in a cowpea genotype from Ghana and South Africa was between 49-157 kg N ha^{-1}. Nitrogen fixation contributed significantly to the sustainability of the cowpea cropping system (Unkovich *et al*., 2008). De Freitas *et al*., (2012), using ^{15}N isotope, showed that cowpea fixed 9-51 kg N ha^{-1} in Brazil, while it was from 12-22 kg N ha^{-1} (Sisworo *et al*., 1990). Studies have also reported that N fixation in sole cropping in Tanzania is about 70 kg N ha^{-1}, while in maize intercropping, it was about 36 kg N ha^{-1} (Vesterager *et al*., 2008). In Zimbabwe, with 1:1 cowpea and cotton intercropping, it was 92 kg N ha^{-1} (Rusinamhodzi *et al*., 2006).

2.9: Factors influencing and affecting BNF

The process of BNF is influenced based on the availability of nutrients. Phosphorus, which is highly important for plant growth and N fixation, depends upon legume-rhizobia symbiosis (Vance, 2001). Potassium is a co-factor of enzymes that alleviate water and environmental stress by improving plant resistance during symbiotic N fixation. In nodule organogenesis, calcium ions (Ca^{2+}) play a vital role in signal translocation (Reddy, 2001). In the early stage of a legume root infection, magnesium (Mg) acts as an enzyme cofactor and binds to rhizobia, stabilizing cell membranes, ribosomes, and nucleic acids (Guo *et al*., 2016). Sulphur deficiency may affect nodule development function and decrease N fixation (Weisany *et al*., 2013). Zinc (Zn) plays a vital role in protein synthesis, and leghaemoglobin and its deficiency reduce nodule size and number. Broadley *et al*., (2012) reported that cobalamin (vit B_{12}) cobalt coenzyme is essential for nodule metabolism, and deficiency leads to decreased N fixation and nodulation.

Besides, Boron plays an indispensable role during nodule formation, cell wall development, and cell division, and its deficiency causes a reduction in growth, size, and nodule number (Weisany *et al*., 2013). The nodulation and N fixation also affect Na and

Cl^- ions (Peoples *et al*., 2009; Dwivedi *et al*., 2015). Salvagiotti *et al*., (2008) reported that the influence of BNF in the agricultural system is due to plant and rhizobia genotype, their management, and the environmental conditions. Some of the factors that limit the BNF include drought, excessive soil moisture, high temperature, pest and disease, nutrient deficiencies, salinity, and toxicity due to soil alkalinity or acidity.

2.10: The occurrence of *Bradyrhizobium* strains in cowpea

The genus *Bradyrhizobium* is well distributed biogeographically and adapted to drier climatic conditions (Koppell and Parker, 2012). Wade *et al*., (2014) reported that *Bradyrhizobium* strain from the drier north part of Senegal increased cowpea nodulation more than the humid south. Gronemeyer *et al*., (2014) reported that Namibia's semi-arid site had diverse rhizobial isolates than moisture-laden areas. Zhang *et al*., (2011) said *Bradyrhizobium* strain distribution in several legumes and suggested that it depends on the influence of P on biological N fixation. Further, P plays a central role in energy metabolism and high nucleic acid content (Cao *et al*., 2014).

2.11: Alternate strategies to increase cowpea production and yield through BNF

The cowpea production in smallholder farms in most parts of the world is limited due to nutrient deficiencies, low and erratic rainfall, and pest (aphids, nematodes, and *Alectra* infection). Frossard *et al*., (2009) reported that subsistence agriculture lacks nutrients, a significant problem in Sub-Saharan Africa. The adaptation of germplasm with high nutrient acquisition efficiency is most important to increase nutrient use efficiency. At the same time, it is essential to identify genotypes with high BNF, superior plant growth, and greater grain yield. Many cowpea genotypes have been evaluated in Ghana and South Africa for their symbiotic N fixation efficiency (Belane *et al*., 2011). Studies from Cameroon and Nigeria reported cowpea genotypes for P use efficiency and BNF (Abaidoo *et al*., 2017). Phosphorus efficient genotype utilization in smallholder farms is essential where P is a limiting factor. AM fungal co-inoculation in cowpea enhances P acquisition. It improved nodulation resulting in improved yield (Andrade *et al*., 2013; Johnson *et al*., 2016).

CHAPTER 3: Detailed survey of *Vigna unguiculata* growing areas and identification of various cultivars from Goa

3.1: INTRODUCTION

Cowpea is native to West Africa, grown primarily in low land and semi-arid regions, and the yield varies based on location (da Silva *et al*., 2018; Pule-Meulenberg *et al*., 2010). The cowpea plant is known to fix 74-116 kg N ha^{-1} per year (Simunji *et al*., 2019). Also, it is drought-tolerant and highly suitable for cultivation in rice fallows. It can be grown on wide soils characteristics such as coarse gravel, sandy, red, heavy loam, and hilly tracts of slopy land (Ramesh *et al*., 2013). In India, cowpea is one of the essential grain legumes with 3.9 million hectares of cultivated area, with 2.21 million tonnes of production and yielding up to 567 kg ha^{-1}.

Cowpea is an economically significant pulse, vegetable, fodder, textile resource, and green manuring crop (Nyaga and Njeru, 2020). The seeds contain 20-40% proteins, 1.8% fats, 60.3% carbohydrates, and rich calcium sources (Majnoon Hoseni, 2008). It is suitable for various cropping systems and is grown in South India, particularly Andhra Pradesh, Tamil Nadu, Karnataka, and Goa. Although numerous high-yielding cowpea varieties are available across India, Goan cowpea (locally known as '*Alsando*') has unique seeds with soft texture, exquisite taste, and bold size. Some local cultivars grown in Goa region include Alsando-1, Goa Nadora (Nadora Bardez-4) and Dhulae Utorda-3 (Goa Cowpea-3) (Manjunath *et al*., 2013).

Biradar *et al*., (2010) reported that the hybridization of Goan cowpea cultivars with selected cultivars like C-152, KM-1, and V-118 showed increased productivity by manipulating biological traits such as photosynthesis and chlorophyll content. Cholin *et al*., (2010) showed genotype M17, Goa, and Bailhongal local as superior stables across all the environments for seed yield. Although the Goan cowpea cultivars exhibited several unique characteristics, it is observed that no extensive survey has been carried out to document the various available cultivars across the state. Hence, the present work was attempted to survey and record the different cowpea cultivars grown in Goa. The work also tried to identify superior high yielding cultivar/s and use them for further research.

3.2: MATERIALS AND METHODS

3.2.1: Study sites

Goa, situated between $15^0$48′00″ N and $14^0$53′54″ N Latitude and $74^0$20′13″E and $73^0$40′ 33″ E Longitude has an area of 3702 sq. km (Gune, 1979). It receives 2800-3000 mm rainfall. The State of Goa which lies on the West Coast of India has favourable factors for increasing many agriculture commodities, particularly horticulture plantation crops. The soils are fertile, and the terrain is undulating. Further, the warm, humid tropical climate favours various flora and fauna for growth and development. The low-lying areas are dominated by cropping systems, *i.e.,* rice and rice-based systems, while the uplands are dominated by cashew and coconut plantations. In Goa, rice-pulse, rice-groundnut, and rice-vegetables methods are practiced. However, the state's productivity levels of vegetables and pulse crops are low compared to national productivity. The crop's cost and yield can be improved by introducing new agricultural and technological inputs.

Cultivars from 18 different agricultural sites in Goa *viz.,* Sangolda-I (S-I), Sangolda-II (S-II), Sangolda-III (S-III), Macasana (S-IV), Moira (S-V), Dadachiwadi Dhargalim (S-VI), Deulwada Harmal (S-VII), Harmal Pernem (S-VIII), Corjuem (S-IX), Coimavaddo Aldona (S-X), Utorda (S-XI), Mencurem (S-XII), Ozarim-I (S-XIII), Ozarim-II (S-XIV), Dulape (S-XV), Colva (S-XVI), Porvorim (S-XVII), and Guirim (S-XVIII), and a released variety *viz.,* Goa cowpea-3 from ICAR-CCARI were taken in the present study.

3.2.2: Collection of cowpea samples

During the study period, field visits were carried out to collect different cowpea cultivars from Goa. In all, 18 cultivars were collected during the field visits. Besides, a local cultivar released by ICAR-CCARI Ella, Old Goa, was also included in the study. Fifty dried pods harvested from the field were used to analyze the yield characteristics. Yield parameters *viz.,* pod length, pod dry weight, number of seeds/pod, seed weight/pod, the weight of 100 seeds, and straw yield were recorded.

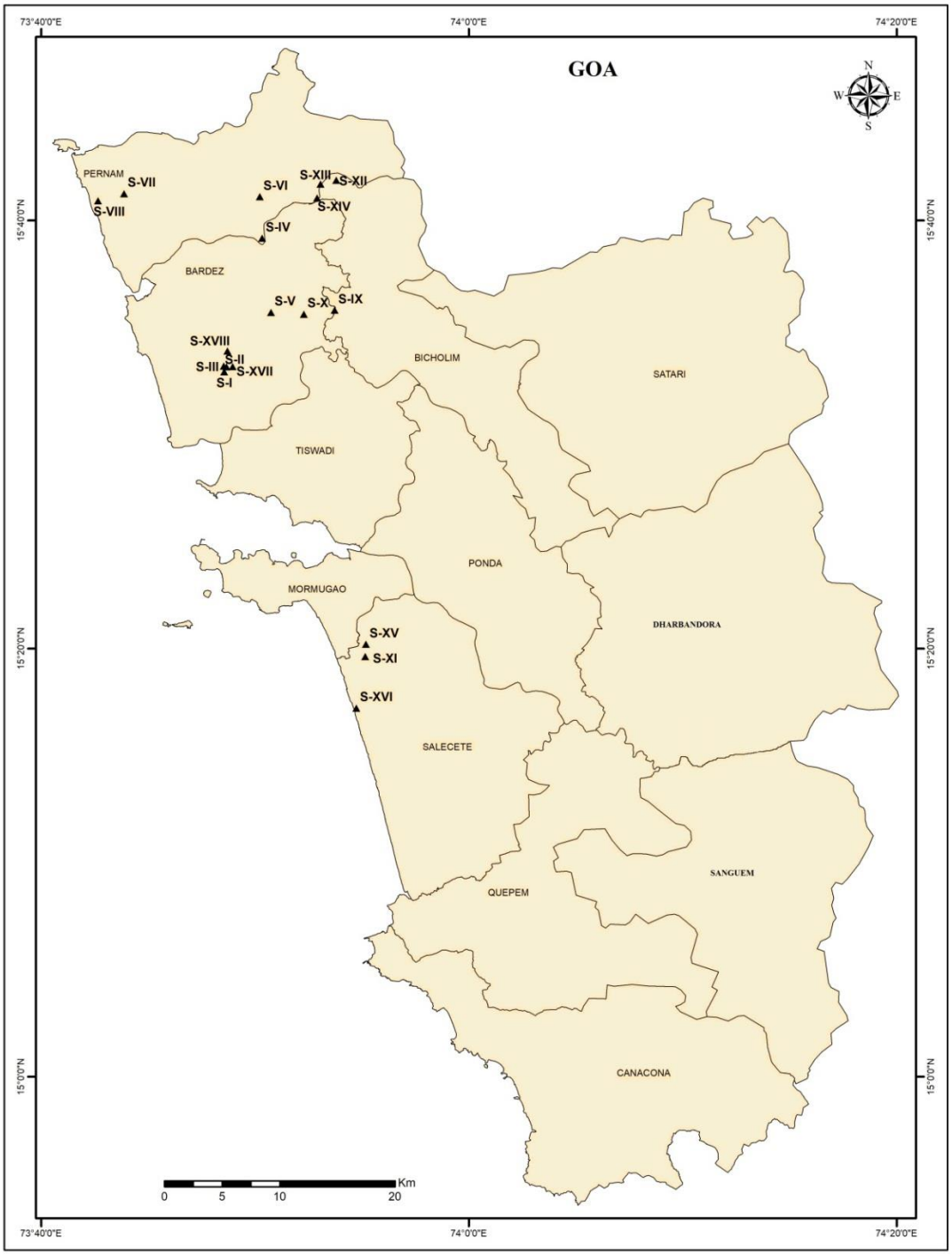


Fig. 3.1: Map of Goa showing study sites.

3.2.3: Cowpea field trial

A field trial was undertaken to compare the yield of 19 cowpea cultivars. A field was selected at Colva village in South Goa. The land was ploughed using the tractor and harrowed after one week. The area was divided into plots of convenient size. Cultivars were sown in plot sizes of 3 × 3 m with 60 × 60 cm spacing and at a depth of 5 cm. Cowpea, in general, are sensitive to waterlogging and require less moisture compared to other vegetables. The plots were irrigated after sowing, at flowering and pod development stages. NPK fertilizer was applied (Urea 1.6 kg, Rock phosphate 3.1 kg, and Muriate of potash 1.9 kg). Timely management practices and plant protection measures were followed to raise a good crop as per the standard recommended practice.

Growth characteristics *viz.*, plant height, leaf number, number of branches, yield, and yield-related parameters were analyzed. The plant height was measured using measuring tape from soil level to terminal bud every month after planting for three months. The number of leaves and branches was determined by visually counting the leaves and branches for each seedling per variety. At maturity, *i.e.* 100-105 days after planting (DAP), pods were harvested by hand, sun-dried for four days, and later shelled. The dry grain yield in each plot per plant was recorded. One hundred seeds were picked from the grains in each cultivar and weighed. Other parameters measured include the number of pods per plant, pod length, number of seeds per pod, number of seeds per plant and seed weight per plant, the weight of 100 seeds, and straw yield.

3.3: RESULTS AND DISCUSSION

3.3.1: Cultivars collected from various agricultural sites

Sangolda

Sangolda is a scenic village situated in Bardez taluka of North Goa. The sites selected in this village include S-I (Sangolda-I, $15^0$32'55.82"N, and $73^0$48'33.32"E), S-II (Sangolda-II, $15^0$33'09.75"N and $73^0$48'40.12"E) and S-III (Sangolda-III, $15^0$33'11.65"N and $73^0$48'32.32"E). In the Rabi season, local cowpea is primarily cultivated in this area under residual moisture conditions.

Macasana

Macasana is a village situated in Pernem taluka of North Goa. Along with local cowpea, groundnuts are also grown in this area. The site selected was S-IV (Macasana, $15^0$39'10.24"N and $73^0$50'19.93"E).

Moira

Moira is a village in Bardez Taluka of North Goa. It is known for its famous bananas. The site S-V (Moira, $15^0$35'42.52"N, and $73^0$50'44.59"E) was selected.

Dadachiwadi Dhargalim

Dadachiwadi Dhargalim village is situated in Pernem taluka of North Goa. Apart from sugarcane cultivation, the local cowpea is grown in this region. The site selected for the study was S-VI (Dadachiwadi Dhargalim, $15^0$41'6.69"N and $73^0$50'13.41"E).

Deulwada Harmal

Deulwada Harmal is situated in Pernem taluka of North Goa. Along with the famous Rabi chilly, local cowpea is also grown. The site selected was S-VII (Deulwada Harmal, $15^0$41'15.49"N and $73^0$43'52.41"E).

Harmal Pernem

Harmal Pernem is situated in Pernem taluka of North Goa. Apart from local vegetables, cowpea is cultivated in the Rabi season. Site S-VIII was selected (Harmal Pernem, 15^0 40'55.60"N Latitude and 73^0 42'39.72"E Longitude).

Corjum

Corjuem village is located in Bardez taluka of North Goa. Mainly local cowpea and chilly are cultivated in this area. The selected site was S-IX (Corjuem, $15^0$35'48.66"N and $73^0$53'43.20"E).

Coimavaddo Aldona

Coimavaddo Aldona is situated in Bardez taluka of North Goa. Site S-X (Coimavaddo Aldona, $15^0$35'37.28"N and $73^0$52'16.78"E) was selected.

Utorda

Utorda village is situated in Salcete taluka of South Goa. In the Rabi season, local vegetables, onion, and watermelon are cultivated. The local cowpea variety, Goa cowpea-3, was released by ICAR-CCARI from Utorda and Dulape areas. The site S-XI (Utorda, $15^0$19'37.36"N and $73^0$55'8.71"E) was selected.

Mencurem

Mencurem is a village in Bicholim taluka in North Goa. Site S-XII (Mencurem, $15^0$41’53.13” N and $73^0$53’47.72” E) was selected.

Ozarim

Ozarim is a village situated in Pernem taluka in North Goa. Site S-XIII (Ozarim-I, $15^0$41’4 .19” N and $73^0$53’03.57” E) and site S-XIV (Ozarim-II, $15^0$41’02.92” N and $73^0$52’53.73” E) were selected. To a lesser extent, most of the fields in this area are cultivated with cowpea and groundnut.

Dulape

Dulape village is situated in Salcete taluka of South Goa. The local cowpea variety, Goa cowpea-3, was released from Utorda and Dulape by ICAR-CCARI. It is one of the significant areas for cowpea production in Goa. Site S-XV (Dulape, $15^0$20’12.16” N and $73^0$55’11.15” E) was selected.

Colva

Colva village is situated at Salcete taluka of South Goa. Site S-XVI (Colva, $15^0$17'13.14"N and $73^0$54'44.05"E) was selected.

Porvorim

Porvorim is situated in Bardez taluka of North Goa. Site S-XVII (Porvorim, $15^0$33'13.84"N and $73^0$48'56.19"E) was selected.

Guirim

Guirim is a village in Bardez taluka of North Goa. Site S-XVIII (Guirim, $15^0$33'45.96"N and $73^0$48'42.16"E) was selected.

Table 3.1: Farmers details of *V. unguiculata* grown cultivar from the agricultural sites.

Sr. No.	Name of the Farmers	Total Area under Cultivation (Sq.mt)	Field/sites	Taluka
1.	Ramchandra Pandurang Kudnekar	2500	Sangolda-I	Bardez
2.	Datta S. Padnekar	4000	Sangolda-II	Bardez
3.	Anuja Anand Salgaonkar	5000	Sangolda-III	Bardez
4.	Vithal L. Kambli	3000	Macasana	Pernem
5.	Laxmi Pandurang Nanuskar	2500	Moira	Bardez
6.	Laxman Narayan Rahut	3500	Dadachiwadi Dhargalim	Pernem
7.	Netaji Naik	2500	Deulwada Harmal	Pernem
8.	Tukaram G. Gaude	2500	Harmal Pernem	Pernem
9.	Janki Ramchandra Parwar	2500	Corjuem	Bardez
10.	Laxmi Kharpal	3500	Coimavaddo Aldona	Bardez
11.	Antones Mendes	2500	Utorda	Salcete
12.	Nita P. Parab	1500	Mencurem	Bicholim
13.	Tukaram G.Naik	2000	Ozarim-I	Pernem
14.	Satyavan K. Pednekar	3000	Ozarim-II	Pernem
15.	Maria Fernandes	3500	Dulape	Salcete
16.	Pitter DCosta	2500	Colva	Salcete
17.	Victor Vaz	1500	Porvorim	Bardez
18.	Avarava Castro	2500	Guirim	Bardez

Table 3.2: Data on pod characteristics of *V. unguiculata* from the farmers' field.

Sites	PL (cm)	PW (g)	S/P	SW/P (g)	SY (g)	SW(g)
S-I	18.27±2.17	2.27±0.79	10.46±3.09	1.69±0.67	0.57±0.36	19.20
S-II	20.85±2.93	3.56±0.87	12.38±2.93	2.74±0.73	0.70±0.22	23.20
S-III	20.56±2.18	3.15±0.58	14.50±2.28	2.39±0.41	0.67±0.19	16.90
S-IV	24.19±3.09	4.57±0.73	12.54±2.32	2.97±0.64	0.81±0.23	26.70
S-V	23.93±3.14	4.18±0.80	13.62±2.36	3.07±0.64	1.01±0.30	24.60
S-VI	21.71±2.49	3.98±1.07	12.58±2.84	3.05±0.95	0.84±0.24	27.30
S-VII	20.65±2.73	3.42±0.72	13.00±2.27	2.65±0.64	0.66±0.22	22.90
S-VIII	19.30±1.50	3.11±0.61	13.12±2.05	2.40±0.50	0.61±0.15	19.60
S-IX	19.44±2.76	3.18±0.78	11.82±2.55	2.60±0.68	0.51±0.18	23.10
S-X	22.73±2.91	3.99±0.82	12.46±2.43	2.96±0.74	0.92±0.22	26.70
S-XI	22.78±2.33	4.39±0.69	12.60±2.24	3.29±0.56	1.02±0.26	27.80
S-XII	23.71±2.43	4.07±0.87	14.36±2.70	2.97±0.68	0.95±0.26	25.60
S-XIII	21.32±1.69	3.87±0.75	13.20±2.02	3.06±0.71	0.77±0.22	26.30
S-XIV	22.47±2.08	4.13±0.86	12.82±2.20	3.16±0.69	0.90±0.25	27.40
S-XV	22.24±2.34	4.39±0.71	12.40±1.83	3.28±0.61	1.01±0.23	26.40
S-XVI	18.36±1.78	2.90±0.58	10.32±1.74	2.23±0.51	0.59±0.15	18.80
S-XVII	17.73±2.42	3.05±2.74	10.38±2.86	1.70±0.60	0.53±0.18	15.30
S-XVIII	18.86±3.43	3.24±0.81	11.04±1.99	2.45±0.70	0.72±0.23	19.50
S-XIX	22.61±2.54	3.98±0.82	15.04±2.56	3.32±0.78	0.61±0.15	28.20

***n=50**

Legend: PL= Pod length (cm), **PW**= Pod dry weight (g), **S/P**= Number of seeds/pod, **SW/P**= Seed weight/pod (g), **SY**= Straw yield (g), **SW**= Weight of 100 seeds (g); Sangolda-I **(S-I)**, Sangolda-II **(S-II)**, Sangolda-III **(S-III),** Macasana **(S-IV),** Moira **(S-V)**, Dadachiwadi Dhargalim **(S-VI)**, Deulwada Harmal **(S-VII)**, Harmal Pernem **(S-VIII)**, Corjuem **(S-IX)**, Coimavaddo Aldona **(S-X)**, Utorda **(S-XI)**, Mencurem **(S-XII)**, Ozarim-I **(S-XIII)**, Ozarim-II **(S-XIV)**, Dulape **(S-XV)**, Colva **(S-XVI)**, Porvorim **(S-XVII)**, and Guirim **(S-XVIII)**, Goa cowpea-3 **(S-XIX)**.

Analysis related to pod characteristics revealed that the cultivar Goa cowpea-3 performed better, and the yield was higher and hence is more suitable for cultivation than other cultivars **(Table 3.2)**.

3.3.2: Field trials

In Goa, cowpea is a significant legume crop cultivated in rice-fallow areas under residual moisture conditions during the rabi season. Based on the local collection, the cultivar was named as per their locality or place of collection. A total of 18 local cultivars were collected from the farmer's field (**Table 1**), and also one ICAR-CCARI release cultivar or variety was used in the present study **(Fig. 1)**. The cultivars collected included Sangolda-I, Sangolda-II, Sangolda-III, Macasana, Moira, Dadachiwadi Dhargalim, Deulwada Harmal,

Harmal Pernem, Corjuem, Coimavaddo Aldona, Utorda, Mencurem, Ozarim-I, Ozarim-II, Dulape, Colva, Porvorim, Guirim **(Plate 3.1; 3.2; 3.3)** and Goa cowpea-3. Further, of the three selected local cultivars *viz.,* Alsondo-l, Goa Nadora (Nadora Bardez-4), and Dhulape Utorda-3 (Goa Cowpea-3) from Goa, only Goa Cowpea-3 was recommended for cultivation (Manjunath *et al*., 2013). Besides, three more promising cowpea cultivars, *viz*., PCP-1131, SKAU-C- 407, and RC 101, have been introduced for cultivation in Goa under rice-fallow conditions for their yield and straw purpose (Manohara *et al*., 2021).

The present study compared the growth and yield of 19 different cultivars with an objective to select the best performing cultivar/s for further research. *Vigna unguiculata* field trial was undertaken at Colva village in South Goa situated at Latitude $15^0$17'12.88"N and Longitude $73^0$54'43.92"E **(Plate 3.4)**. The results indicate that for the various parameters measured, the Goa Cowpea-3 (ICAR-CCARI Goa) cultivar showed better growth and yield performance than the other cultivars tested **(Table 3.3 Plate 3.5; 3.6; 3.7)**. This cultivar recorded a higher number of pods per plant (24.44), seeds per pod (12.74), seeds per plant (313.44), seed weight per plant (86.63 g), and weight of 100 seeds (26.10 g) higher as compared to other cultivars. The study revealed that the different cultivars varied considerably in seed size, shape, and colour **(Plate 3.8; 3.9; 3.10)**.

Further, the yield data of 50 pods from the farmer's field and the field trial data confirmed that the Goa cowpea-3 cultivar was superior to all other cultivars. Therefore, the Goa cowpea-3 cultivar was selected for the experimental trial, which is reported in Chapter 9 of the thesis. However, most farmers in Goa prefer the local varieties due to their tolerance to moisture stress and semi-spreading nature. Besides, farmers fetch a premium price for their produce due to their taste, bold size seeds, and better cooking quality (Manohara *et al*., 2021).

The present study observed that the cultivar Goa cowpea-3 is grown only in Utorda and Dulape regions of Salcete taluka in South Goa. The farmers grow a*lsando* at the onset of the winter season as this would lead to demand and would favour early harvesting of the crop (Manjunath *et al*., 2013). In North Goa, areas covered under cowpea cultivation include Macasana, Ozarim, Guirim, and Sangolda, while in South

Table 3.3: Vegetative and yield characteristics of various cowpea cultivars undertaken for the study.

Sites	PHt (cm)	L/P	PrB	DFF	PL (cm)	P/P	S/P	S/PLt	SW/PLt (g)	SY (g)	Weight of 100 seeds (g)
S-I	83.55±35.39	23.00±4.96	3.15±1.15	55.00±0.00	18.43±1.83	12.78±6.96	10.25±1.50	125.33±60.60	34.39±15.36	58.70	17.10
S-II	59.40±34.53	25.04±10.25	3.52±1.15	60.00±0.00	16.89±2.65	6.11±2.13	8.30±2.50	51.78±22.08	17.54±5.37	35.80	19.20
S-III	47.48±26.16	24.41±6.29	3.56±0.98	57.00±0.00	19.45±19.45	14.44±3.34	10.30±0.91	148.56±34.19	32.24±5.75	70.30	14.30
S-IV	54.32±20.05	35.56±10.75	4.85±0.69	54.00±0.00	22.88±5.94	11.89±4.41	9.40±0.80	113.56±48.04	30.46±10.44	80.40	23.50
S-V	51.03±22.47	36.15±12.27	3.56±1.54	59.00±0.00	23.09±1.72	7.33±3.40	9.68±0.94	68.67±26.99	22.23±7.86	60.60	21.40
S-VI	55.03±17.35	41.37±11.30	4.30±1.28	55.00±0.00	19.93±2.16	15.78±5.51	9.37±1.90	145.22±47.37	41.02±11.28	135.30	24.20
S-VII	35.59±7.88	24.07±7.23	3.33±1.01	61.00±0.00	18.74±2.66	8.00±2.16	9.35±1.90	73.44±19.71	23.60±4.91	35.20	18.70
S-VIII	57.66±14.96	33.56±7.88	3.81±1.30	60.00±0.00	18.70±2.39	19.33±4.92	11.13±0.69	199.67±58.93	52.34±14.13	115.30	17.20
S-IX	65.86±20.41	58.22±16.01	5.15±1.06	55.00±0.00	17.86±1.05	21.67±5.37	10.27±1.06	226.22±73.22	58.07±16.97	120.40	20.10
S-X	66.67±19.42	53.48±15.45	4.96±1.35	65.00±0.00	21.68±1.58	16.00±6.38	11.21±3.17	177.22±74.36	48.19±18.73	100.20	23.20
S-XI	64.53±22.51	50.67±10.13	5.33±1.15	58.00±0.00	19.52±1.63	20.56±8.39	9.26±1.47	197.22±103.47	61.41±28.93	108.30	24.80
S-XII	51.18±17.67	38.00±14.68	4.37±1.51	59.00±0.00	21.38±1.05	18.11±6.81	11.54±1.28	211.22±89.93	55.57±20.49	140.20	22.30
S-XIII	79.71±27.06	43.22±10.48	4.89±0.59	60.00±0.00	17.96±2.27	13.11±4.09	8.98±2.05	119.44±52.69	35.43±14.95	80.50	23.10
S-XIV	81.71±29.25	46.37±9.70	5.11±1.38	65.00±0.00	20.40±1.99	13.33±4.57	10.58±1.45	142.67±52.22	43.93±15.29	90.60	22.50
S-XV	72.32±19.89	50.19±7.33	5.56±0.67	60.00±0.00	20.27±1.78	23.33±7.35	9.68±1.77	265.11±112.58	81.18±36.13	110.20	23.10
S-XVI	49.72±12.06	37.48±9.07	3.70±1.10	65.00±0.00	18.50±1.65	9.33±3.02	10.30±1.40	95.74±34.57	28.97±9.43	60.30	15.20
S-XVII	58.24±11.52	37.33±6.93	3.93±0.95	65.00±0.00	16.97±2.06	10.78±1.62	10.27±1.14	111.25±22.44	36.08±8.13	54.40	12.10
S-XVIII	50.76±9.19	27.88±5.50	3.74±1.74	60.00±0.00	16.17±1.96	10.00±2.11	9.94±0.90	99.40±23.17	31.78±4.01	69.30	15.40
S-XIX	58.20±12.41	56.74±12.73	**5.33**±0.80	55.00±0.00	21.82±1.00	**24.44±7.69**	**12.74±1.02**	**313.44±107.47**	**86.63±27.75**	105.20	**26.10**

Legend: DFF= Days for flowering, **PHt**= Plant height (cm), **PrB**= Primary branches/plant, **L/P**= Number of leaves/plant, **P/P**= Number of pods/plant, **P/L**= Pod length (cm), **S/P**= Number of seeds/pod, **S/PLt**= Number of seeds/plant, **SW/PLt**= Seed weight/plant (g), **SY**= Straw yield (g), Sangolda-I **(S-I)**, Sangolda-II **(S-II)**, Sangolda-III **(S-III)**, Macasana **(S-IV)**, Moira **(S-V)**, Dadachiwadi Dhargalim **(S-VI)**, Deulwada Harmal **(S-VII)**, Harmal Pernem **(S-VIII)**, Corjuem **(S-IX)**, Coimavaddo Aldona **(S-X)**, Utorda **(S-XI)**, Mencurem **(S-XII)**, Ozarim-I **(S-XIII)**, Ozarim-II **(S-XIV),** Dulape **(S-XV)**, Colva **(S-XVI)**, Porvorim **(S-XVII)**, and Guirim **(S-XVIII)**, Goa cowpea-3 **(S-XIX)**.

Goa, it is in Utorda and Dulape. Besides commercial crops, cowpea is considered one of Goa's economic rabi legume crops for small-scale farmers.

3.4: CONCLUSION

Besides other vegetable crops, the cowpea is considered an economically important rabi pulse crop in Goa. It is successfully cultivated on the sandy silt soils of the region. The cultivars differ from place to place in pod length, seed number, size, colour, and yield. Even with a place-to-place variation, the farmers prefer the local cultivar based on the availability and choice of seed material. Based on the demand and easy management, crop production increases every year. The farmers prefer cultivating different local cultivars under residual moisture conditions for higher income. Even though many cowpea hybrid varieties are available, farmers prefer the local cowpea due to its bold size and unique taste.

CHAPTER 4: Physico-chemical characteristics of *V. unguiculata* grown soils

4.1: INTRODUCTION

Soil is an essential resource for plant growth. Its physical, chemical, and biological characteristics largely determine the plants that can grow on them. The soil's physical characteristics include soil texture, structure, consistency, and water holding capacity (Gardner *et al*., 1999). Further, the electrical conductivity (EC), pH, N, P, and K, and other macro- and micro-elements describe the chemical characteristics of the soil (Nabiollahi *et al*., 2017, Li *et al.*, 2013; Ngo-Mbogba *et al.,* 2015). In India, large-scale legume crop production may be severely affected by the soil's texture, organic carbon content, and chemical composition (Nyabyenda, 2005). While assessing the impact of soil on plant growth, Ossom and Rhykerd (2007) noted that the development and plant growth largely depends on soil's chemical properties and mineral nutrient concentration. Thus, soil fertility plays a crucial role in increasing legume productivity in agriculture.

The soils of the coastal region of Goa are lateritic (81%), containing a high amount of iron and aluminium oxides and hydroxides. The soils are primarily sandy loam to silt loam texture, well-drained, and acidic (5.5-6.5 pH) (Mahajan *et al*., 2015). Cowpea, a drought-tolerant crop, can grow on many soils but prefers sandy, infertile, and acid soils. The cowpea plant enriches soil fertility through biological nitrogen fixation (Kamara *et al*., 2017). In the present chapter Physico-chemical characteristics of soils from cowpea growing agricultural fields were analyzed.

4.2: MATERIALS AND METHODS

4.2.1: Soil sampling and analysis

Rhizosphere soil samples were collected from 18 dominant cowpea growing agricultural sites in Goa. Random sampling was followed, which would be more suitable for a study area. Fifteen plant-soil samples were collected from each site. These samples were mixed thoroughly to obtain a composite sample from each site to ensure homogeneity among replicated samples. The samples were collected in clean, sterile polyethylene bags and air-dried under controlled laboratory conditions. The Physico-chemical analysis of the soil samples was carried out at ICAR - Central Coastal Agricultural Research Institute Ela, Old

Goa. The air-dried samples were gently crushed using mortar and pestle and sieved through 2 mm and 0.5 mm sieves before being used for laboratory analyses. The soil pH and EC were measured in 1:2.5 soil to water suspension using pH and EC meter, respectively (Jackson 1973). The soil organic carbon (OC) was determined using Walkley and Black's (1934) wet-oxidation method. Nitrogen in the soil was estimated by the alkaline potassium permanganate method (Subbiah and Asija 1956) and P by colorimetric method (Bray and Kurtz 1945). Available potassium (K) was estimated using 1N ammonium acetate solution through Atomic Absorption Spectrophotometric (AAS) analysis (Hanway and Heidel 1952). Other micronutrients *viz.*, zinc (Zn), copper (Cu), manganese (Mn), and iron (Fe) were analyzed by diethylenetriaminepentaacetic acid extraction method (Lindsay and Norwell 1978) using Atomic Absorption Spectrophotometer (AAS) (nova 400P, Analytik Jena, Germany).

4.2.2: Determination of Soil Texture

Soil texture determination was carried out using the Pipette analysis method (Folk, 1980). Oven-dried sediment (15 g) was taken in a 1000 mL beaker. Distilled water was added, and the sediment was stirred and allowed to settle. The following day the water was decanted. The above steps were repeated 4-5 times to remove salinity. After this, 10mL of 10% hexametaphosphate and 5 mL of 30% H_2O_2 were added to the sediment sample. The sediment sample was then poured through a 63 µm sieve. The filtrate was collected in a 1000 mL measuring cylinder. The sediment sample on the sieve was then washed till the filtrate became a clear solution. The filtrate volume was 1000 mL with distilled water in the measuring cylinder.

The resultant solution was homogenized for about 2 minutes at room temperature using a stirrer for pipette analysis. 25 mL solution was pipetted out from the measuring cylinder, transferred in a pre-weighed beaker, dried at 60^0C overnight, and then weighed to determine the clay weight. The sand remaining on the 63µm sieve was transferred to a pre-weighed beaker and dried at 60^0C overnight, after which the content was weighed (sand weight).

Percentage of sand, silt and clay were calculated using the formulae given below:

% Sand = (wt. of the sand/total wt.) x 100

% Clay = (wt. of clay/total wt.) x 100

% Silt = 100- (% of sand + % of clay)

4.3: RESULTS AND DISCUSSION

4.3.1: Soil Physico-chemical analyses

The results of Physico-chemical analyses of the soils are depicted in (**Table 4.1**). The soil pH ranged from 4.72-5.94, indicating the acidic nature. Although the soil pH was lower than 6.0, it showed significant variations. It was least at S-XVIII and highest at S-VII. Mahajan *et al*., (2015) suggested that the soil pH does not alter salinity but increases soluble salts. Therefore, soil pH is a vital requirement in sustainable agriculture for improving crop production (Kumar *et al*., 2013). Nabiollahi *et al*., (2017) suggested that pH and EC are used as soil indicators for chemical characteristics. Besides, the soil pH influences cowpea bradyrhizobial diversity (Wang *et al*., 2016).

The EC of soils from various study sites ranged from 0.02-0.65 ds m^{-1}, suggesting their non-saline nature. The variation in the EC values in acidic soil may be due to the high amount of Fe and Al oxides and hydroxides. According to Mahajan *et al*., (2015), heavy rainfall is known to wash out salts resulting in decreased EC. Goa receives more than 3000 mm of rain per year, spanning over 5-6 months. Low EC maintains soil moisture content, bulk density, porosity, hydraulic conductivity, and soil water retention potential (Mahajan *et al*., 2020), making the land suitable for rice, followed by cowpea cultivation.

The soil organic carbon (OC) varied significantly at different sites and ranged from 0.09-0.95%. Most of the study sites showed medium to high OC content. The soil microflora maintains organic matter and soil aggregate formation (Sardinha *et al*., 2003), while a high level of organic carbon helps increase soil productivity and sustain soil health (Singh, 2006). Organic matter plays an essential role in water-holding, increased cation exchange capacity, soil aggregates stability, and micronutrient retention.

The present study revealed variations in macro-and micro-nutrients at the different study sites. The available soil N, P, and K at various locations ranged from 100.35-288.98 kg ha^{-1}, 6.55-12.16 kg ha^{-1}, and 65.45-191.42 kg ha^{-1}, respectively. Available N was recorded in low amounts, whereas P and K recorded low to medium amounts in the soils. Significantly higher levels of available N (288.98 kg ha^{-1}) were recorded at the S-XI site. Further, at four locations, S-IV (10.01 kg ha^{-1}), S-VII (10.13 kg ha^{-1}), S-XII (12.16 kg ha^{-1}), and S-XVI (11.08 kg ha^{-1}) medium P levels were recorded. At the remaining sites, P content in the soil

was low, attributed to soil acidity (**Fig 4.1)**. Low available P content may be due to Fe and Al oxides and hydroxides in the soil (Mahajan *et al*., 2016). Potassium (K) was recorded in low to medium concentrations in the soil. Besides being an exchangeable cation and a chemical indicator for soil quality, K enhances salinity stress in plants (Sharma *et al*., 2008; Ngo-Mbogha *et al*., 2015).

Essential nutrients such as Fe, Mn Cu, and Zn are used as basic soil indicators for soil quality assessment (Nabiollahi *et al.,* 2017). In the present study, DTPA-extractable micro-nutrients (Zn, Fe, Mn, and Cu) were found at varying concentrations in the soil. The available micro-nutrients Zn (0.09-0.96 mg kg^{-1}), Fe (8.43-19.31 mg kg^{-1}), Mn (12.33-28.99 mg kg^{-1}) and Cu (0.20-0.71 mg kg^{-1}) varied in the soils. The values reported in the present study are lower than those reported by Mahajan *et al*., (2015) for coastal acidic soils. The study revealed that the soils' Fe, Zn, and Cu levels were within limits while they were low in Mn. The micronutrient levels of the soil are known to affect soil pH and soil texture. It also affects soil microbial activity (Sardinha *et al.,* 2003). It is a known fact that the soil nutrient status and nutrient supply capacity is essential for a sustainable agricultural management system (Powlson *et al*., 1987).

Soil texture and higher organic C affect legume grain production (Nyabyenda, 2005). The soil texture results of the present study have been elaborated through a ternary diagram, as depicted in **Fig 4.2**. The results indicated that in most cases, the soils had a high percentage of sand (mean= 57.16%) followed by silt (mean= 42.16%), while clay percentage was the least (mean= 0.68%) (**Table 4.2**). These results conform with an earlier study (Mahajan *et al.,* 2016). Clay content in soils was higher in inland soils, while coastal soils were rich in sand and silt.

Table 4.1: Physico-chemical analysis of *V. unguiculata* growing soils.

Site	pH	EC (dS m^{-1})	Organic Carbon (%)	Macro-nutrients (kg ha^{-1})			Micro-nutrients (mg kg^{-1})			
				N	P	K	Zn	Fe	Mn	Cu
S-I	5.00±0.01	0.05±0.01	0.75±0.01	253.00±3.14	8.11±0.12	86.30±0.80	0.14±0.01	17.48±1.12	26.40±0.82	0.36±0.02
S-II	4.95±0.02	0.13±0.01	0.95±0.03	255.13±2.92	6.93±0.44	170.35±1.07	0.14±0.01	11.70±0.71	28.99±1.27	0.51±0.01
S-III	4.93±0.01	0.03±0.01	0.78±0.02	260.38±4.16	6.60±0.53	191.42±1.16	0.74±0.02	12.47±1.26	16.61±0.46	0.66±0.03
S-IV	5.44±0.06	0.07±0.01	0.30±0.02	217.42±1.77	10.01±0.01	158.39±0.93	0.09±0.01	18.11±0.99	12.43±0.80	0.71±0.01
S-V	5.24±0.04	0.02±0.01	0.73±0.03	200.35±2.20	8.33±0.87	142.69±1.17	0.13±0.01	18.41±1.16	13.16±1.09	0.28±0.02
S-VI	4.80±0.02	0.06±0.01	0.60±0.02	247.06±1.74	8.51±0.31	171.33±0.02	0.44±0.01	19.31±1.09	12.33±1.13	0.47±0.01
S-VII	5.94±0.02	0.18±0.02	0.53±0.01	223.00±0.63	10.13±0.03	109.00±1.00	0.13±0.01	14.15±0.54	14.36±0.89	0.38±0.01
S-VIII	4.85±0.01	0.20±0.01	0.67±0.03	250.32±0.12	8.42±0.62	173.11±0.99	0.13±0.01	9.66±0.58	13.40±1.04	0.47±0.02
S-IX	5.35±0.01	0.02±0.01	0.60±0.03	225.44±1.00	7.99±0.27	88.77±1.21	0.30±0.01	8.43±0.20	17.00±1.12	0.75±0.01
S-X	4.75±0.01	0.29±0.01	0.79±0.01	214.33±0.56	8.31±0.70	94.11±1.00	0.75±0.01	12.00±0.67	16.00±1.01	0.35±0.01
S-XI	4.92±0.02	0.06±0.01	0.61±0.01	288.98±0.89	8.22±1.00	97.13±1.04	0.11±0.01	10.05±1.04	14.47±1.32	0.69±0.02
S-XII	5.69±0.05	0.20±0.01	0.95±0.02	263.42±0.69	12.16±1.00	86.46±0.64	0.96±0.01	16.16±1.04	23.19±1.01	0.24±0.01
S-XIII	5.60±0.02	0.45±0.01	0.19±0.01	238.34±1.15	8.57±1.11	65.45±0.51	0.45±0.01	13.23±0.95	15.40±1.18	0.62±0.01
S-XIV	5.67±0.05	0.12±0.01	0.53±0.01	175.62±0.71	9.24±1.00	114.02±1.00	0.47±0.01	12.47±0.92	15.60±0.69	0.20±0.01
S-XV	5.17±0.02	0.65±0.01	0.50±0.02	100.35±0.46	8.66±1.19	70.78±1.11	0.11±0.01	19.69±1.01	23.28±1.07	0.35±0.01
S-XVI	5.69±0.03	0.09±0.01	0.09±0.01	112.90±0.94	11.08±1.05	85.95±0.31	0.95±0.02	11.82±0.94	21.57±1.02	0.35±0.01
S-XVII	5.37±0.03	0.07±0.01	0.63±0.03	262.17±0.52	9.01±1.01	89.40±1.02	0.25±0.01	12.99±0.31	17.14±0.96	0.38±0.01
S-XVIII	4.72±0.02	0.05±0.01	0.48±0.01	265.30±0.82	6.55±0.79	182.44±1.21	0.11±0.01	8.46±0.60	14.32±1.03	0.28±0.01

Legend: *All values are mean of three replicates; Study sites: **S-I** = Sangolda-I, **S-II** = Sangolda-II, **S-III** = Sangolda-III, **S-IV** = Macasana, **S-V** = Moira, **S-VI** = Dadachiwadi Dhargalim, **S-VII** = Deulwada Harmal, **S-VIII** = Harmal Pernem, **S-IX** = Corjuem, **S-X** = Coimavaddo Aldona, **S-XI** = Utorda, **S-XII** = Mencurem, **S-XIII** = Ozarim-I, **S-XIV** = Ozarim-II, **S-XV** = Dulape, **S-XVI** = Colva, **S-XVII** = Porvorim, and **S-XVIII** = Guirim.

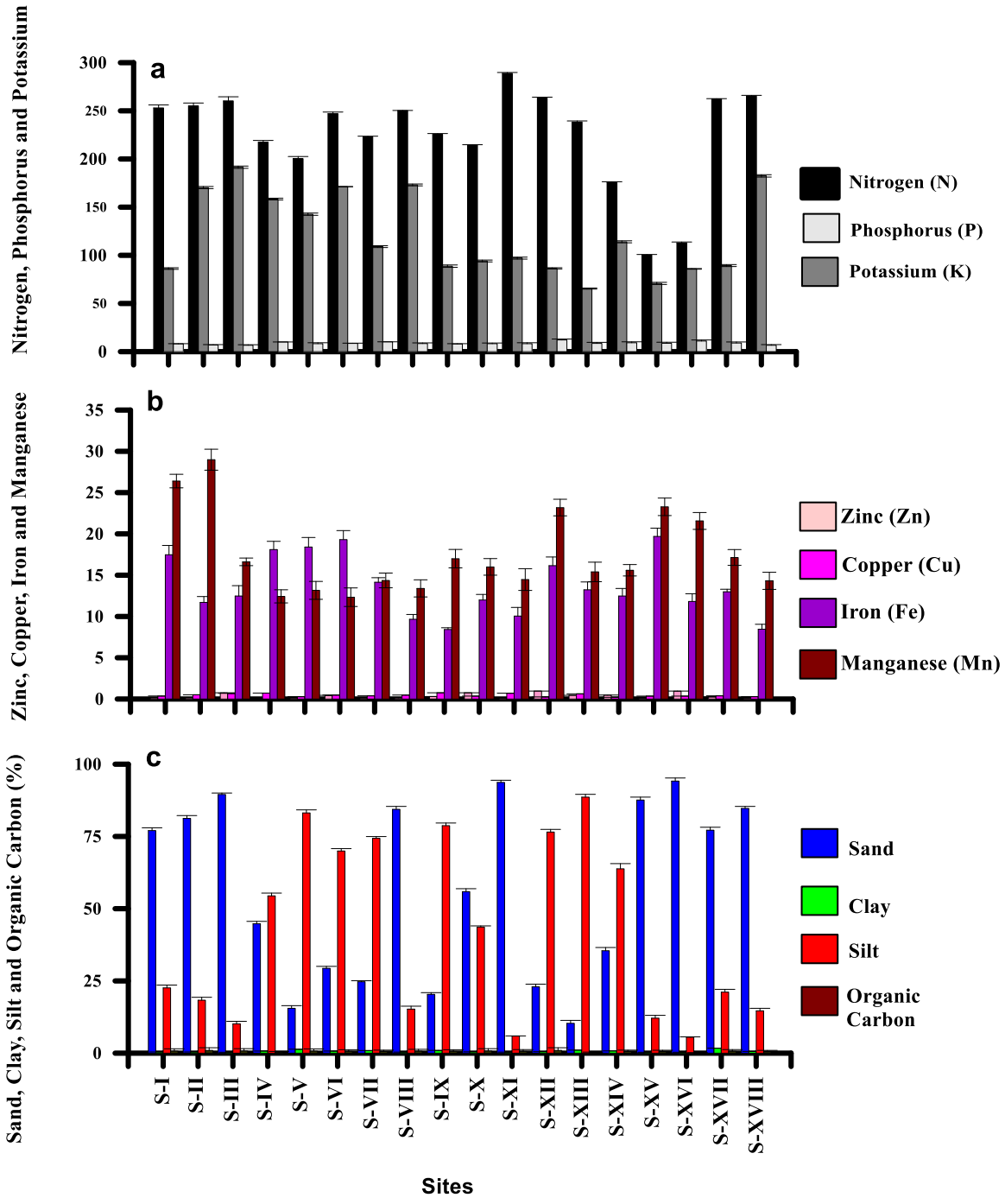


Fig 4.1: Variations in Physico-chemical properties of soils from various study sites. a. N, P, and K; **b.** Zn, Cu, Fe, and Mn; **c.** Sand, silt, clay, and organic carbon.

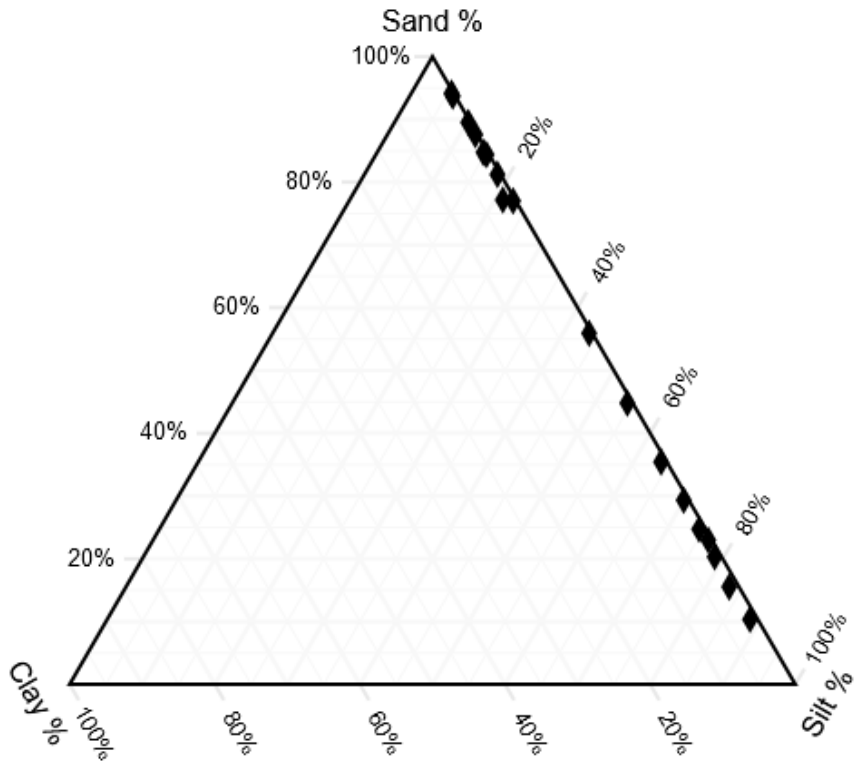


Fig. 4.2: A ternary diagram of the soil texture from the study sites.

Table 4.2: Soil texture from the study sites.

Site	% Sand	% Silt	% Clay
S-I	77.01 ± 1.00	22.60 ± 0.99	0.39 ± 0.01
S-II	81.25 ± 0.98	18.33 ± 1.03	0.43 ± 0.01
S-III	89.53 ± 0.50	10.16 ± 0.87	0.31 ± 0.02
S-IV	44.81 ± 0.83	54.45 ± 0.95	0.74 ± 0.01
S-V	15.51 ± 0.95	83.14 ± 1.07	1.35 ± 0.01
S-VI	29.35 ± 0.71	69.95 ± 0.82	0.70 ± 0.01
S-VII	24.72 ± 0.36	74.39 ± 0.57	0.89 ± 0.02
S-VIII	84.38 ± 1.01	15.26 ± 1.00	0.36 ± 0.01
S-IX	20.34 ± 0.62	78.72 ± 0.95	0.94 ± 0.01
S-X	55.91 ± 1.00	43.64 ± 0.44	0.46 ± 0.01
S-XI	93.72 ± 0.73	5.94 ± 0.05	0.34 ± 0.02
S-XII	22.98 ± 0.91	76.52 ± 0.92	0.50 ± 0.01
S-XIII	10.33 ± 1.00	88.60 ± 0.95	1.07 ± 0.01
S-XIV	35.44 ± 1.10	63.79 ± 1.79	0.77 ± 0.01
S-XV	87.58 ± 1.04	12.11 ± 1.00	0.31 ± 0.01
S-XVI	94.17 ± 1.05	5.53 ± 0.11	0.30 ± 0.02
S-XVII	77.18 ± 0.98	21.11 ± 1.01	1.71 ± 0.02
S-XVIII	84.71 ± 0.68	14.68 ± 0.85	0.61 ± 0.01
Mean	**57.16%**	**42.16%**	**0.68%**

Legend: ± = Standard deviation; All values are mean of three replicates.

4.4: CONCLUSION

The Physico-chemical soil properties of the study sites indicated that the soil in these areas is acidic, with pH ranging from 4.7 to 5.9. It contains low to medium levels of available N, P, and K. The soils were deficient in DTPA-extractable micronutrients. The texture of the soil was mainly sandy loam with the minimum amount of clay present in it. It is concluded that the soil pH, EC, and micronutrients are the most critical soil properties controlling its quality in agricultural areas.

CHAPTER 5: Study on the AM fungal diversity in different cultivars of *V. unguiculata*

5.1: INTRODUCTION

Vigna unguiculata L. (Walp), the most prominent pulse rich in protein, starch, iron, phosphorus, calcium, and vitamin B, is being cultivated in 45 countries worldwide. Of the total world production, 84% comes from sub-Saharan Africa (Abate *et al*., 2011). In India, cowpea is a popular legume pulse component of the intercrop farming system. In many studies, cowpea being a leguminous plant has been selected as a scavenger crop due to the broad host range of rhizobial diversity. Cowpea legume is a multilateral symbiosis with N-fixing rhizobia and P-acquiring AM fungi (Scheublin *et al*., 2004). Biological nitrogen fixation (BNF) enhances soil fertility and N-status in cowpea, reducing N fertilizer application (Martins *et al*., 2003).

The productivity and diversity of crops in a particular region are based on the diversified function of AM fungal symbiosis, which can influence all metabolic processes and significantly improve plant growth and development (Lone *et al*., 2017). Modern intensive agricultural practices such as chemical fertilization and pest control, continuous monoculture, and tillage impact the AM fungi and plant interactions. Therefore, AM fungal diversity in an area is essential for determining the effects of agricultural treatments upon AM fungi. The eventual development of management regimes of these fungi for sustainable agriculture would help minimize the use of chemical fertilizers and biocides.

In an agricultural ecosystem, the distribution, identification, quantification, and diversity of AM fungi are essential for understanding the role of plant, fungus, and soil interaction. In a sustainable agroecosystem, the role of AM fungi is vital to understanding their potential involvement in productivity (Khalil *et al*., 1992). Identifying AM fungal soil community based on spore hyphae, intra-radical hyphae, vesicles, and quantification of diversity and dominance is essential to knowing the biota of the soil community. AM fungi play a crucial role in soil dynamics, diversity, and plant community structure. Plant nutrition, water uptake, and resistance to soil-borne and plant diseases prevail over biotic and abiotic stresses. N nutrient cycling, growth survival, and uptake of soil nutrients, especially P, acquire a vital role in C and N cycle and contribute to C sinks (Douds *et al*., 2005; Smith and Read, 2008; Turrini and Giovannetti, 2012). AM fungal studies in

cowpea are scarce even though it is a highly economically important crop of the agro-ecosystem (Tawaraya, 2003). Based on spore morphology, the diversity of AM fungi in cowpea has been studied in Benin (Johnson *et al*., 2013). The diversity of native AM fungal species in cowpea grown agricultural soils of Goa is less known.

Soil texture, mineral composition, decomposition, and organic matter mineralization limit agricultural production. Organic carbon in the soil is the composition of the degraded form of organic matter. It plays a crucial role in maintaining the soil's physical, chemical, and biological properties (Micheni *et al*., 2004). AM fungal mycelium plays a vital role in decomposing organic matter and facilitates nutrient uptake from available resources (Hodge *et al*., 2010). In an ecosystem, AM fungal root colonization, diversity and sporulation patterns of AM fungal genera and species, and spore numbers are determined based on soil nutrient properties (Muthukumar and Udaiyan, 2000). Further, at different P-level, cowpea cultivars may vary in response to colonization by AM fungi and plant promoting growth due to more excellent absorption of P by extra-radical hyphae (Smith and Read, 2008).

Cowpea has a significant mycotrophic status with the occurrence of arbuscules and vesicles in the roots. However, cowpea and AM fungus exhibit a preferential symbiosis (Johnson *et al*., 2016). The AM fungal distribution is affected by many biotic and abiotic factors such as host plant species, pH, ecosystem type, moisture and temperature, total C and N content, season, and disturbance regime (Boddington and Dodd, 2000; Carvalho *et al*., 2003). However, some factors influencing the distribution and assessing the diversity are mandatory in ecology. AM fungal community structure was significantly recognized in crop cultivation, and roots act as bio-fertilizers for their eventual valorization. Hence, the present study is aimed to assess the diversity of indigenous AM fungal species associated with the rhizosphere of some dominant cowpea cultivars grown at agricultural sites in Goa.

5.2: MATERIALS AND METHODS

5.2.1: Samples collection

The rhizosphere soil and root samples from18 study sites were collected from 0-20 cm depth. The soil was collected from five randomly selected locations (from the vicinity of 15 cowpea plants) at each site. Soils collected from each site were mixed to form a

composite sample from which three replicate sub-samples were drawn. The soil samples were packed in polyethylene bags, labeled, brought to the laboratory, and stored in a deep freezer at 4^0C until analyzed.

5.2.2: Processing of root segments for AM fungal colonization

Trypan blue staining technique (Phillips and Hayman, 1970) was employed to evaluate AM colonization in roots. The roots were washed with tap water, cut into 1 cm segments, and cleared in 5% KOH (90^0C for 45 minutes in the oven), thoroughly rinsed in tap water, acidified with 5N HCl for 5 minutes, and drained. To this, 0.05% Trypan blue stain was added and kept overnight. The stained root segments were examined using bright-field Olympus BX 41 (4X-100X) and Nikon Eclipse E200 research microscopes. Micrographs were imaged using Nikon Digital Sight DS-U3 digital camera. The root segments were checked for the presence or absence of AM hyphae, arbuscules, vesicles, hyphal coils, and auxiliary cells.

5.2.3: Estimation of AM fungal colonization

Estimation of AM colonization was carried out using the Root Slide method (Read *et al*., 1976). The Trypan blue stained root fragments were mounted in polyvinyl-Lacto-glycerol (PVLG), and the presence or absence of AM colonization was scored using the following formula:

Root colonization (%) = (No. of root segments colonized/Total no. of root segments scored) x 100

5.2.4: Isolation of AM fungal spores

AM fungal spore isolation was carried out using the wet sieving and decanting method (Gerdemann and Nicolson, 1963). 100 g of rhizosphere soil sample was placed in a beaker, and tap water was added. The soil suspension was stirred continuously using a glass rod, and the sediment was allowed to settle for 1 minute. This aliquot was decanted through sieves arranged in descending order (250-37µm). The procedure was repeated twice for each sample. The residues from each sieve were then washed into separate beakers. The aliquot was filtered separately through Whatman No. 1 filter paper. The filter paper was then placed in a Petri plate to ensure that it remained moist. The filter paper was examined for the presence of spores and sporocarps under Olympus stereo microscope SZ2-ILST (10 x 4.5 zoom).

5.2.5: Taxonomic identification of spores

Clean, intact, and un-parasitized spores were used for taxonomic identification and quantification. Intact and crushed spores, mounted in Poly Vinyl-Lacto Glycerol (PVLG) (Koske and Tessier, 1983), were examined under Olympus BX 41 microscope. Taxonomic identification of AM spores was carried out based on morphological characteristics using relevant bibliographies (Morton and Benny, 1990; Schenck and Perez, 1990; Almeida and Schenck, 1990; Rodrigues and Muthukumar, 2009; Schüßler and Walker, 2010; Blaszkowski, 2012; Redecker *et al*., 2013), and online species matching the descriptions provided by the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (https://invam.wvu.edu/).

5.2.6: Determination of AM species richness, Spore abundance, Relative abundance, and Isolation frequency

AM fungi's species richness, spore abundance, and relative abundance (RA%) were calculated using the following formulae (Beena *et al*., 2000c, 2001).

5.2.6.1: Species richness: Species number per 100 g soil sample or species number per study site.

5.2.6.2: Spore abundance: Number of spores of a particular species per 100 g of soil sample.

5.2.6.3: Relative abundance (RA%) = (Number of spores of a particular species/total number of spores) x 100

5.2.6.4: Isolation frequency (IF%) = (Number of soil samples containing particular species/total number of soil samples analyzed) x 100

5.2.7: Diversity Studies

AM fungal diversity studies were carried out at the selected sites in North and South Goa. Species richness is the number of species present in an ecosystem. Simpson's diversity index of AM fungal species diversity and abundance was calculated using Simpson's Diversity index *1- D* (Simpson, 1949), $D=1-\Sigma\ (Pi)^2$ where $Pi = ni/N$, (ni) the relative abundance of the species, is calculated as the proportion of individuals of a given species

(*ni*) to the total number of individuals in a community (*N*). Simpson's reciprocal index was calculated using the following formulae, 1/D**.** Shannon diversity index (*H*) is commonly used to characterize species diversity in a community, which accounts for both abundance and evenness of the species present, H= −Σ (*P i*ln (*Pi*) (Shannon and Weaver 1949). Species evenness is calculated by the following formulae, *E* (*H*)= $H/H_{\max}$ where, H_{max}= ln*S, S*= total number of species in the community (richness).

5.2.8: Statistical analysis

All statistical analyses were performed using SPSS 22.0 (IBM, Armonk, NY, USA). A Canonical Correlation Analysis (CCA) using all observations was performed to obtain the interrelationships between the variables studied. Pearson's correlation coefficient was used to find out variation between AM fungal spore density (SD), isolation frequency (IF), relative abundance (RA), and species richness (SR) using variance (ANOVA) and Principal Component Analysis (PCA) using WASP software (Web-Based Agricultural package) 2.0 ($p \leq 0.05$).

5.3: RESULTS AND DISCUSSION

5.3.1: Arbuscular mycorrhizal fungi

From the field samples, 9544 AM fungal spores accounting for 22 species belonging to seven genera and three families were recovered **(Plate 5.1; Plate 5.2)**. The highest species richness was recorded in *Acaulospora* (8), *Gigaspora* (4), *Glomus* (4), *Scutellospora* (2), *Funneliformis* (2) *Racocetra* (1), and *Rhizoglomus* (1). Even though the genus *Acaulospora* was dominant, *Gigaspora decipiens* was the most abundant AM species recovered from all 18 study sites. Among the AM fungal species, *Gi. decipiens* followed by *Gi. margarita* dominated the community and were widespread in their distribution **(Fig. 5.1)**. The species composition, diversity, evenness, and richness are depicted in **Fig. 5.2**. The dominance of *Gi. decipiens* and *Gi. margarita* is attributed to the acidic nature of the soil.

5.3.2.: Percent root colonization and spore density of AM fungi

Arbuscular mycorrhizal fungal root colonization was observed at all the 18 dominant cowpea grown agricultural sites. The colonization reported the presence of intra-radical and extra-radical hyphae, hyphal coils, arbuscules, auxiliary cells, and vesicles (**Table 5.3;**

Plate 5.3 & Plate 5.4). AM fungal species showed significant colonization in cowpea at the sampling sites. Maximum root colonization (97.33%) was recorded at S-VII, while minimum (78%) was recorded at S-XVIII. Higher colonization may be attributed to high porosity and less fertility of the sandy, loamy soil.

An earlier study by Dessai and Rodrigues, (2012) reported root colonization in *Vigna unguiculata* from Goa in an earlier study. According to Ouallal *et al*., (2018) these soils offer optimum conditions for mycorrhization. Also, the colonization patterns could result from differences in habitat, soil distribution patterns, and host plant interaction (Hindumati and Reddy, 2011). AM mycorrhization depends on plant species, soil type, and available P (Gaur and Purshotam, 2011).

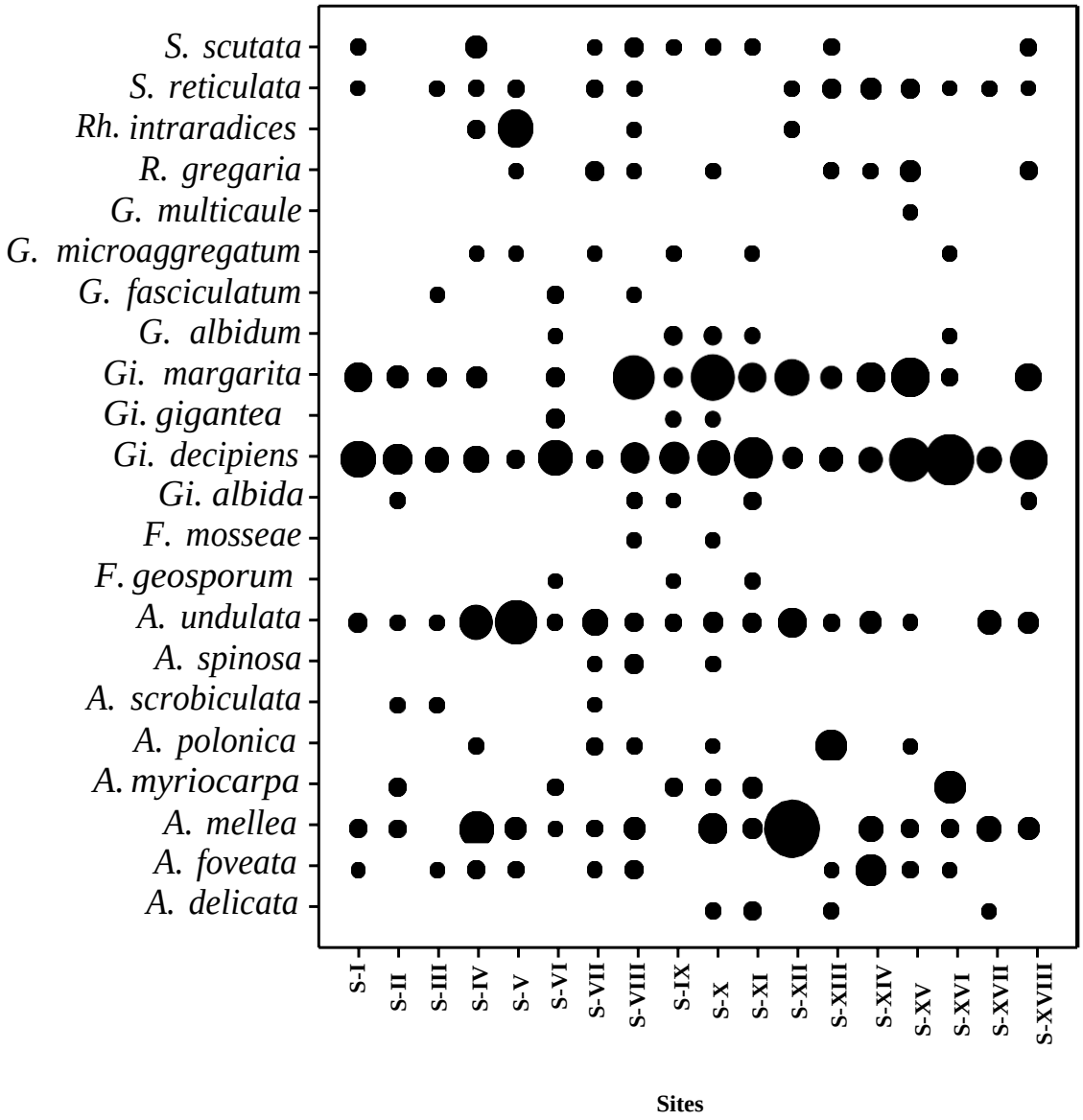


Fig. 5.1: AM fungal community composition (based on mean spore density) at various study sites. (The minimum diameter of 0.05 circles denotes one spore, and a maximum diameter of 0.30 represents 134 spores).

In cowpea, AM fungal association plays a vital role in N and P cycles through Biological Nitrogen Fixation (BNF) (Pontes *et al*., 2017). In the present study, the K level was moderate, which is often a prerequisite for root colonization (Gamage *et al*., 2004) **(Table 4.1)**. Further, root colonization and spore germination depend upon the micro-nutrient availability, which triggers or suppresses the development of mycelial growth (Alguacil *et al*., 2016). AM fungi effectively increase macro-nutrient uptake compared to micro-nutrient and improve plant growth and development (Cwala *et al*., 2010; Lehmann *et al*., 2014).

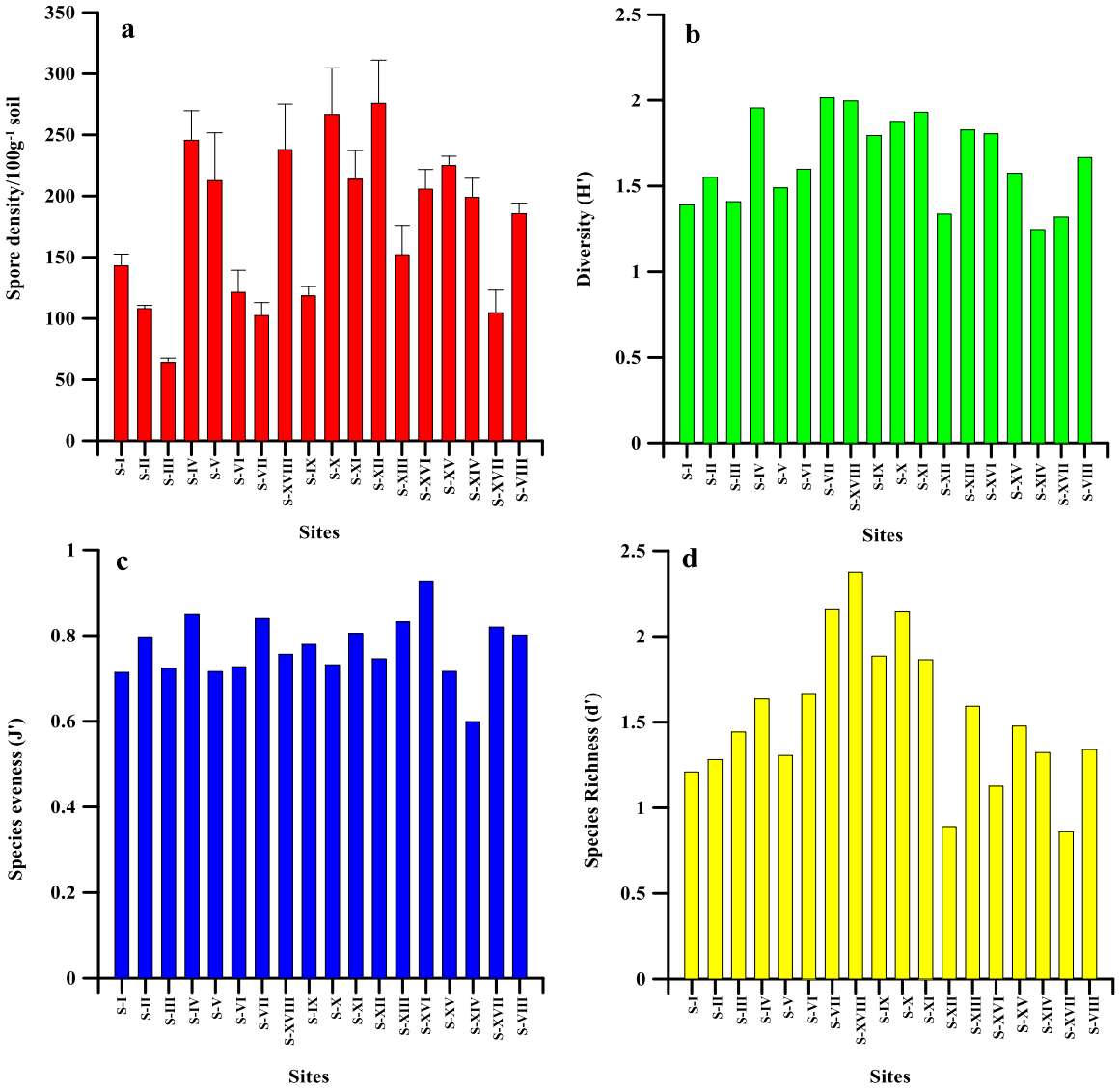


Fig. 5.2: Spatial variations in (a.) Mean cell density, (b.) Species diversity, (c.) Species evenness, and (d.) Species richness.

In the present study, results of spore density exhibited extreme variation. The highest spore density was recorded at S-XII (276 spores 100 g^{-1}), while the minimum was recorded at S-III (64 spores 100 g^{-1}) **(Fig. 5.2 & Fig. 5.4; Table 5.3)**. In sandy, loamy agricultural sites, low spore density may be attributed to site-specificity, soil management, agricultural practice, and environmental stress (Maun, 2009). The highest spore density was recorded in Gigasporaceae (259 spores 100 g^{-1}), followed by Acaulosporaceae (149 spores 100 g^{-1}), and least in Glomeraceae (25 spores 100 g^{-1}) **(Fig. 5.3)**. This follows previous reports, which suggest that in acidic soils (pH 3.9-5.6), species of Gigasporaceae were found more frequently (Picone, 2000; Yano and Takaki, 2005). Acaulosporaceae and Glomeraceae,

although occurring in acidic soils, are also known to prefer alkaline soils (Sati and Tiwari, 2008).

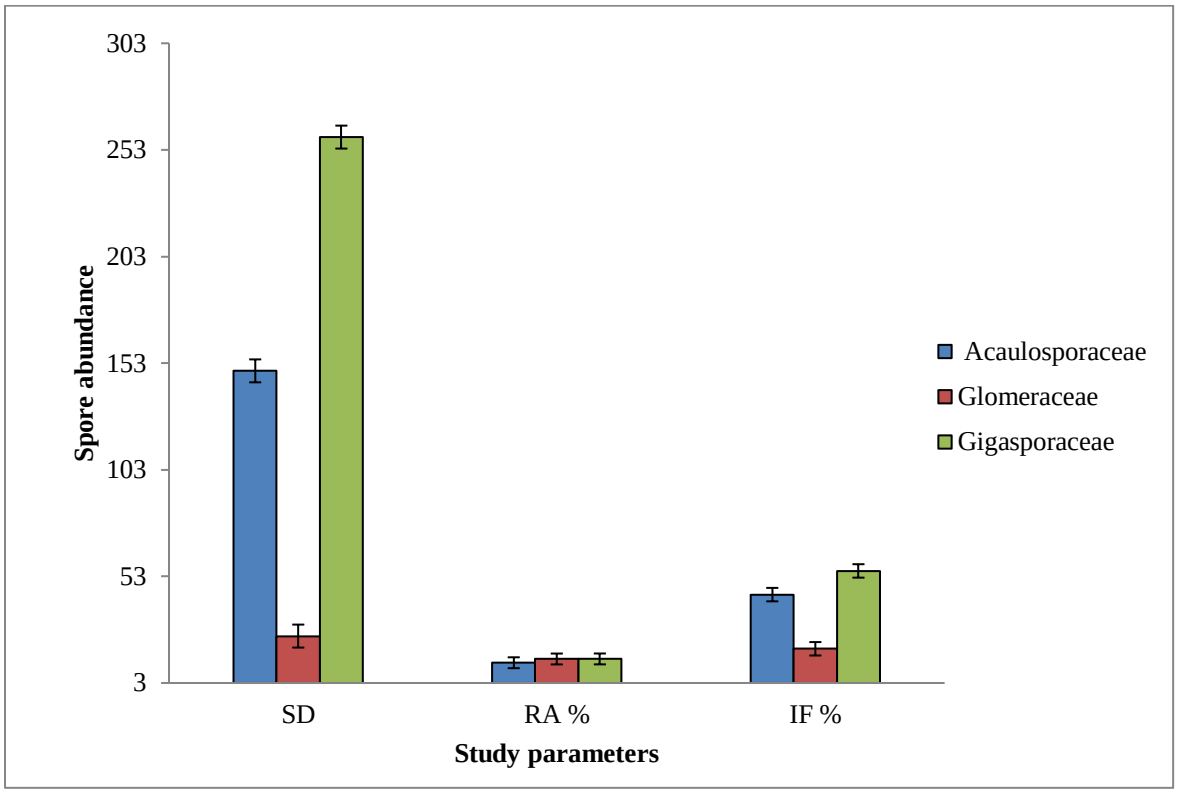


Fig. 5.3: Familywise data on Spore Density (SD), Relative Abundance (RA%), and Isolation Frequency (IF%) at the study sites.

Gigaspora decipiens ranked highest in spore abundance and relative abundance indicating its wide distribution at the study sites **(Table 5.5)**. While *Gigaspora, Acaulospora*, *Glomus,* and *Scutellospora* are utmost widespread in dune vegetation and coastal areas of the world (Willis *et al*., 2013; Maun, 2009). Reports on Gigasporaceae show a predominance in sandy-subtropical to tropical soils (Lee and Koske, 1994; Lekberg *et al*., 2007). From the present study, it has been observed that Gigasporaceae produced more spores than Acaulosporaceae and Glomeraceae. This may be attributed to low soil acidity, sandy loam texture, and different agricultural site-specificity favouring spore germination and sporulation. Soil pH regulates AM spore germination (Maun, 2009). Also, AM fungal communities depend upon micro-climatic changes, *viz.*, soil pH, organic matter, and texture content (Ji *et al.*, 2012; Njeru *et al*., 2015). They also differ within and between species and genera levels due to their natural competitive approach (Horn *et al*., 2014).

5.3.4: AM fungal species richness

The AM fungal species richness was maximum (14 spp) at S-VIII and minimum (5 spp each) at S-XVII and S-XII **(Fig. 5.4)**. In the present study, habitation and landscape patterns differed in sampling sites. An agricultural region's habitat and landscape patterns are known to affect AM fungal richness and composition (Wubet *et al*., 2004). AM species richness depends upon the existing host plant, mycorrhization, and sporulation (Mueller, 2011). The land use pattern, soil properties, and environmental conditions differ with AM fungal taxa (Begoude *et al*., 2016). Further, earlier studies have reported that AM fungal species richness in natural forests was much higher than in agricultural soils (Tchabi *et al*., 2008; de Carvalho *et al*., 2012).

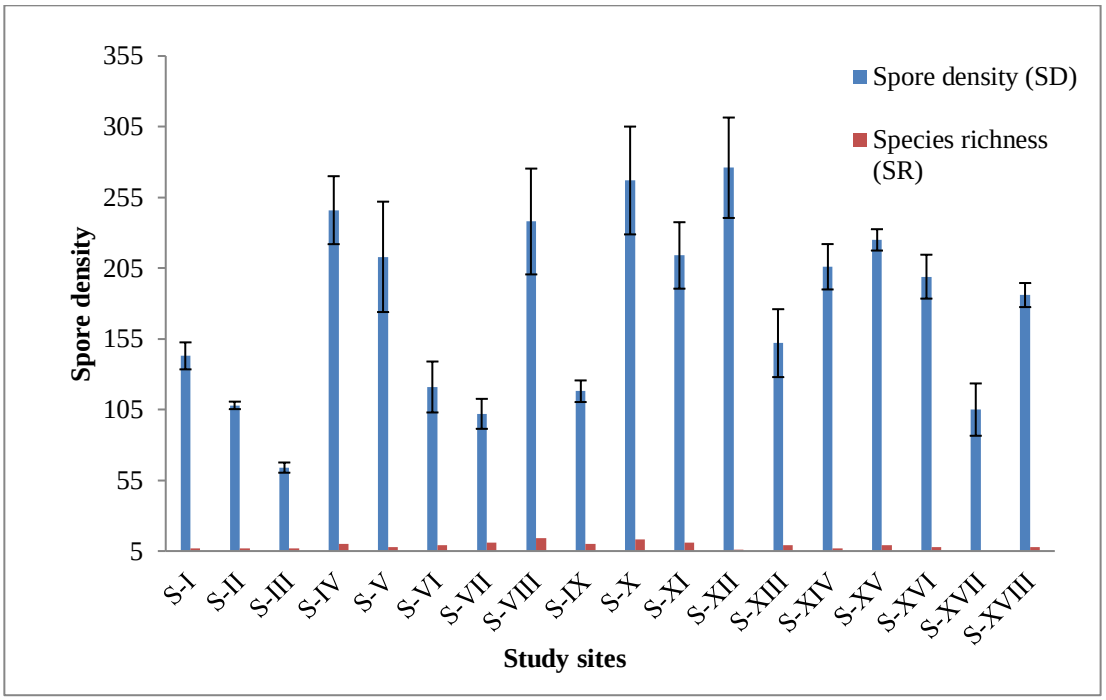


Fig. 5.4: AM spore density and species richness in the study sites.

In the present study, 22 AM fungal species have been reported from 18 different sampling sites. Castillo *et al*., (2006) recorded 22 species belonging to *Glomus*, *Acaulospora*, and *Scutellospora* from an agricultural ecosystem. Johnson *et al*., (2013) recovered 15 AM fungal species from sub-humid Guinea, Savanna, and semi-arid Sudan Savana. Similarly, 21 AM species were reported from NE Brazil and 24 species in Brazilian semi-arid (Sousa *et al*., 2012). Also, Dessai and Rodrigues (2012) reported 51 AM fungal species from 10 different agricultural sites in Goa.

In Brazil, sustainable agriculture using coconut fibre and cow dung manure in the fields recorded high AM species richness (Pontes *et al*., 2017). Lower soil pH recorded a low number of AM fungal species (Mbogne *et al*., 2015). According to Pontes *et al*., (2017), the variation in sporulation may be attributed to biotic and abiotic factors.

5.3.5: Influence of environmental characteristics on the distribution of AM fungi

AM fungal abundance and environmental characteristic probable correlation was performed using the Canonical correspondence analysis (CCA). The arrows in the CCA plot indicate relative significance affecting the community, whereas the angle between variables suggests a degree of correlation **(Fig. 5.5)**. The CCA biplot of AM fungi species explains results on the 4-axis (**Table 5.1**). AM fungal species *viz.*, *Glomus albidum, G. fasciculatum, G. microaggregatum, Rhizoglomus intraradices, Gigaspora gigantea, Acaulospora delicata, A. spinosa,* and *Funneliformis mosseae* showed a positive correlation with organic carbon at S-VI, S-IX, S-XI, and S-X. *Acaulospora scrobiculata, A. myriocarpa, Gigaspora gigantea, Gi. albida, Gi. margarita, Gi. decipiens,* and *Glomus microaggregatum* showed the influence of Mn and EC (ds/m) at S-II, S-III, and S-XVI.

The combination of Fe, Cu, and K influenced the occurrence of *Funneliformis geosporum, F. mosseae, Acaulospora polonica, A. delicata,* and *A. spinosa* at S-V, S-VII, S-I, S-XVII, and S-XII. On the 4th axis, pH, Zn, N, and P correlated with sites S-VIII and S-XIII and AM species *viz.*, *Acaulospora delicata, A. spinosa,* and *Scutellospora scutata*.

Table 5.1: Cumulative constrained percentages of the four-axis extracted in the CCA analysis for AM fungi species.

Factors	Axis 1	Axis 2	Axis 3	Axis 4
AM species	28.23	44.87	60.33	70.68

The Principal Component Analysis (PCA) was carried out to find the influence of parameters on the environment in the study area. In PCA, the Eigenvalues were used to determine the number of principal components (PCS) to be maintained for further study. A scree plot for the Eigenvalues indicated a distinct slope change mainly after the fourth Eigenvalues. Hence, the first four PCs were retained, indicating 68.3% of the variance **(Table 5.2; Fig. 5.6).** Also, the relationship between AM fungal colonization and physicochemical characteristics using Principal Component showed positive scoring.

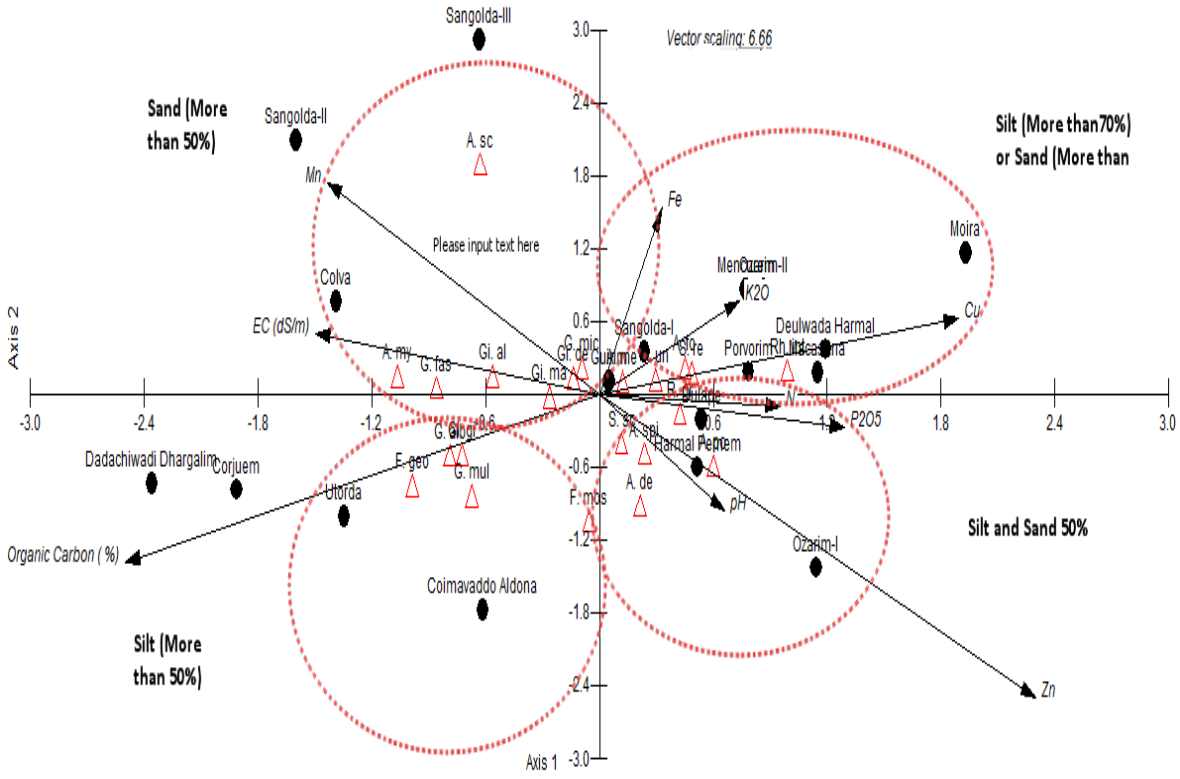


Fig. 5.5: Canonical Correspondence Analysis (CCA) of the relation between the physico-chemical variables. (pH, EC, OC, N, P, K, Zn, Fe, Mn, and Cu) are indicated by arrows.

5.3.5: Relative Abundance (RA) and Spore Abundance (SA)

The Relative Abundance (RA) ranged from 0.54% at site S-XVIII to 55.56% at site S-XVI **(Tables 5.4)**. In high acidic soil, some AM species show lower RA (Bivoko *et al*., 2013; Mbogne *et al*., 2015). However, in sandy loam soils, the highest number of spore counts have been recorded (Muchoka *et al*., 2020). The RA depends on host plant species and ecosystem regions (Radhika and Rodrigues, 2010; Torrecillas *et al*., 2012; Pontes *et al*., 2017). Variations in SA were observed at the study sites. The highest SA was recorded at S-XII, while the lowest SA was recorded at S-IX and S-XVIII. Only four species showed >10% abundance, while 18 species showed <5% spore abundance in the study sites.

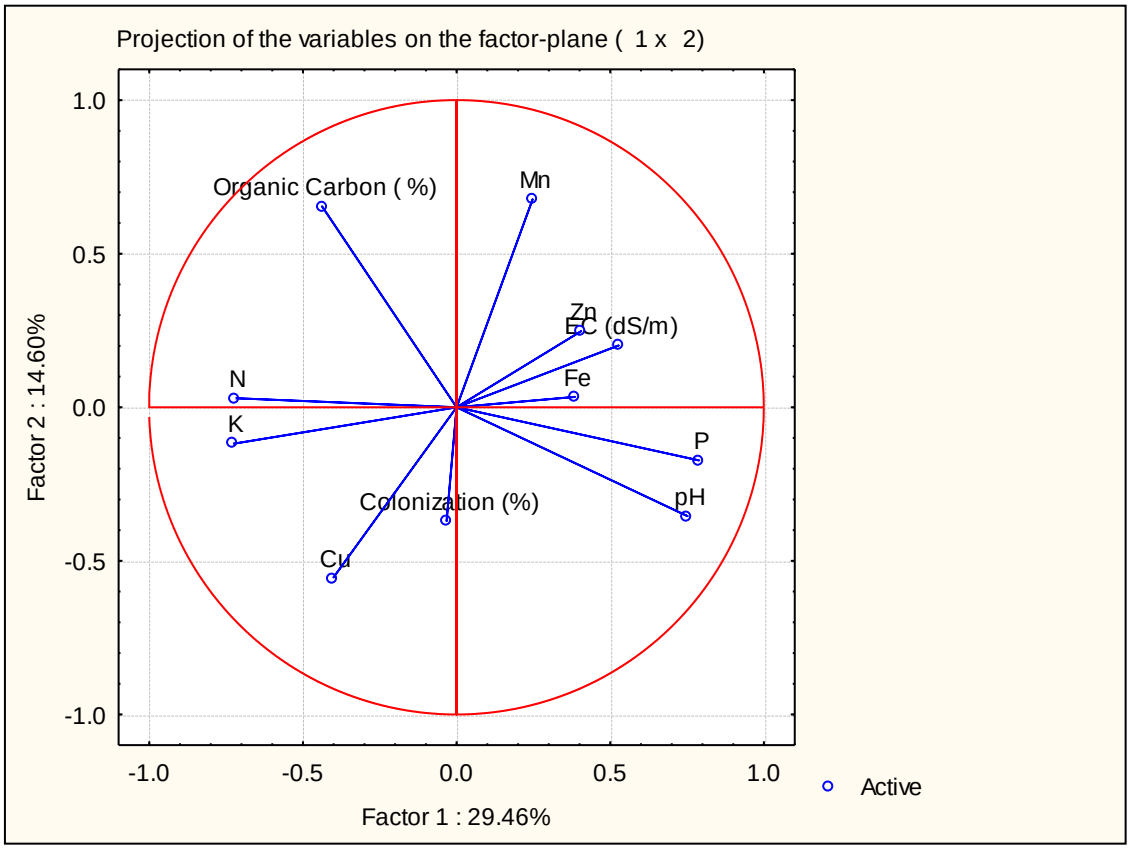


Fig. 5.6: Principal component analysis (PCA) of the relationship between AM colonization and Physico-chemical characteristics in cowpea grown agricultural soils.

The Principal component analysis (PCA**)** test results are presented in **Table 5.6.** The results indicate that the mean spore density showed a significant relationship (P≤0.05) with pH, P, and Cu, whereas species richness with EC, species evenness with Mn, and species diversity with Zn and Cu. However, pH was significant (P≤0.05) with N, P, K, and AM fungal density. Moreover, AM fungal distribution and community structure depends on several factors such as nutrient availability, climatic factors, spatial and temporal variation, and differential sporulation ability of AM fungal species (Muthukumar and Udaiyan, 2002; Renker *et al*., 2005).

Table 5.2: Principal Component Analysis (PCA) of eigenvalues of the correlation matrix and related statistics.

Variables	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
pH	3.241	29.459	3.241	29.459
EC (dS/m)	1.606	14.602	4.847	44.061
Organic Carbon (%)	1.537	13.971	6.383	58.032
N	1.328	12.070	7.711	70.102
P	1.003	9.122	8.715	79.224
K	0.684	6.216	9.398	85.440
Zn	0.619	5.631	10.018	91.070
Fe	0.430	3.913	10.448	94.984
Mn	0.241	2.187	10.689	97.171
Cu	0.189	1.721	10.878	98.892
Colonization (%)	0.122	1.108	11.000	100.000

Legend: EC, **pH**, Organic Carbon **(OC)**, Nitrogen **(N)**, Phosphorus **(P)**, Potassium **(K)**, Zinc **(Zn)**, Iron **(Fe)**, Manganese **(Mn)**, and Copper **(Cu)**.

Table 5.3: AM fungal association in *V. unguiculata* at the study sites.

Study sites	AM fungal species	Type of colonization			Colonization (%)	Spore density	Species richness
		H	A	V			
S-I	*Gi. de, Gi. ma, A. me, A. un, S. sc, S. re, A. fo*	+	+	+	90.00 ±3.46	143 ±9.54	7
S-II	*G. alb, Gi. ma, A. my, A. me, A. un, Gi. al, A. sc.*	+	+	+	90.00 ±5.29	108 ±2.65	7
S-III	*Gi. de, Gi. ma, G. fas, A. un, S. re, A. fo, A. sc.*	+	+	+	90.67 ±4.16	64±3.61	7
S-IV	*Gi. de, Gi. ma, G. mic, Rh. int, A. me, A. un, A. po, S. sc, S. re, A. fo*	+	+	+	93.33 ±7.02	246 ±24.03	10
S-V	*Gi. de, G. mic, Rh. int, A. me, A. un, R. gr, S. re, A. fo*	+	+	+	84.00 ±8.00	213±39.02	8
S-VI	*Gi. de, Gi. ma, F. geo, G. alb, G. fas, A. my, Gi. gi, A. me, A. un*	+	+	+	95.33 ±2.31	121 ±18.01	9
S-VII	*Gi. de, G. mic, A. me, A. un, A. po, R. gr, S. sc, S. re, A. fo, A. sc., A. spi,*	+	+	+	97.33 ±1.15	102 ±10.6	11
S-VIII	*Gi. de, Gi. ma, G. fas, Rh. int, A. me, A. un, A. po, Gi. al, R. gr, S. sc, F. mos, S. re, A. fo, A. spi*	+	+	+	94.00 ±3.46	238 ±37.4	14
S-IX	*Gi. de, Gi. ma, F. geo, G. alb, G. mic, A. my, Gi. gi, A. un, Gi. al, S. sc*	+	+	+	92.67 ±5.77	118 ±7.64	10
S-X	*Gi. de, Gi. ma, G. alb, A. my, Gi. gi, A. me, A. un, A. po, A. de, R. gr, S. sc, F. mos, A. spi*	+	+	+	78.67 ±4.16	267 ±38.11	13
S-XI	*Gi. de, Gi. ma, F. geo, G. alb, G. mic, A. my, A. me, A. un, A. de, Gi. al, S. sc*	+	+	+	82.67 ±8.33	214 ±23.50	11
S-XII	*Gi. de, Gi. ma, Rh. int, A. me, A. un, S. re*	+	+	+	88.67 ±2.31	276 ±35.50	6
S-XIII	*Gi. de, Gi. ma, A. un, A. po, A. de, R. gr, S. sc, S. re, A. fo*	+	+	+	81.33 ±8.33	152 ±24	9
S-XIV	*Gi. de, Gi. ma, A. me, A. un, R. gr, S. re, A. fo*	+	+	+	84.67 ±3.06	206 ±16.04	7
S-XV	*Gi. de, Gi. ma, A. me, A. un, A. po, R. gr, S. re, A. fo, G. mul*	+	+	+	90.00 ±7.21	225 ±7.55	9
S-XVI	*Gi. de, Gi. ma, G. alb, G. mic, A. my, A. me, S. re, A. fo*	+	+	+	84.67 ±6.11	199 ±15.52	8
S-XVII	*Gi. de, A. me, A. un, A. de, S. re*	+	+	+	88.00 ±8.72	105 ±18.50	5
S-XVIII	*Gi. de, Gi. ma, A. me, A. un, Gi. al, R. gr, S. sc, S. re*	+	+	+	78.00 ±10.58	186 ±8.50	8

Legend: ***Gi. de*** *= Gigaspora decipiens,* ***Gi. ma*** *= Gigaspora margarita,* ***F. geo*** *= Funneliformis geosporum,* ***G. alb*** *= Glomus albidum,* ***G. fas*** *= Glomus fasciculatum,* ***G. mic*** *= Glomus microaggregatum,* ***Rh. int*** *= Rhizoglomus intraradices,* ***A. my*** *= Acaulospora myriocarpa,* ***Gi. gi*** *= Gigaspora gigantea,* ***A. me*** *= Acaulospora mellea,* ***A. un*** *= Acaulospora undulata,* ***A. po*** *= Acaulospora polonica,* ***A. de*** *= Acaulospora delicata,* ***Gi. al*** *= Gigaspora albida,* ***R. gr*** *= Racocetra gregaria,* ***S. sc*** *= Scutellospora scutata,* ***F. mos*** *= Funneliformis mosseae,* ***S. re*** *= Scutellospora reticulata,* ***A. fo*** *= Acaulospora foveata,* ***A. sc*** *= Acaulospora scrobiculata,* ***A. spi*** *= Acaulospora spinosa,* ***G. mul*** *= Glomus multicaule.*

Table 5.4: Relative abundance (%) of AM fungal species at the study sites.

Sr. No.	AM species	Study sites																	
		S-I	**S-II**	**S-III**	**S-IV**	**S-V**	**S-VI**	**S-VII**	**S-VIII**	**S-IX**	**S-X**	**S-XI**	**S-XII**	**S-XIII**	**S-XIV**	**S-XV**	**S-XVI**	**S-XVII**	**S-XVIII**
1	*Acaulospora delicata*	nd	nd	nd	nd	nd	nd	nd	0.00	nd	2.37	5.14	nd	3.29	nd	nd	nd	2.54	nd
2	*Acaulospora foveata*	1.17	nd	2.60	5.15	3.60	nd	4.58	5.32	nd	nd	nd	nd	2.19	24.60	2.52	1.34	nd	nd
3	*Acaulospora mellea*	7.69	8.95	nd	25.61	11.11	1.93	6.54	9.94	nd	17.23	7.79	48.55	nd	15.86	5.19	6.03	30.48	12.54
4	*Acaulospora myriocarpa*	nd	10.80	nd	nd	nd	6.61	nd	nd	9.60	2.87	8.72	nd	nd	nd	nd	27.64	nd	nd
5	*Acaulospora polonica*	nd	nd	nd	2.44	nd	nd	8.50	2.10	nd	0.75	nd	nd	35.53	nd	1.33	nd	nd	nd
6	*Acaulospora scrobiculata*	nd	2.78	7.29	nd	nd	nd	2.29	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
7	*Acaulospora spinosa*	nd	nd	nd	nd	nd	nd	3.59	6.16	nd	1.37	nd	nd	nd	nd	nd	nd	nd	nd
8	*Acaulospora undulata*	10.26	3.40	5.73	24.25	41.31	4.41	35.95	5.46	7.34	5.99	7.01	16.18	5.26	11.00	2.07	nd	28.89	11.11
9	*Funneliformis geosporum*	nd	nd	nd	nd	nd	1.65	nd	nd	0.85	nd	2.18	nd	nd	nd	nd	nd	nd	nd
10	*Funneliformis mosseae*	nd	nd	nd	nd	nd	nd	nd	0.98	nd	1.00	nd	nd	nd	nd	nd	nd	nd	nd
11	*Gigaspora albida*	nd	6.79	nd	nd	nd	nd	nd	2.38	1.13	nd	4.21	nd	nd	nd	nd	nd	nd	3.58
12	*Gigaspora decipiens*	45.45	44.44	50.00	14.91	5.63	50.96	10.13	19.75	42.94	22.35	36.76	7.49	19.08	15.70	38.96	55.28	33.33	40.14
13	*Gigaspora gigantea*	nd	nd	nd	nd	nd	12.12	nd	nd	5.37	1.87	nd	nd	nd	nd	nd	nd	nd	nd
14	*Gigaspora margarita*	30.07	22.84	26.04	8.81	nd	13.77	nd	37.25	13.56	35.46	20.56	23.67	16.45	20.71	32.74	5.70	nd	20.79
15	*Glomus albidum*	nd	nd	nd	nd	nd	2.48	nd	nd	12.15	4.87	2.96	nd	nd	nd	nd	1.17	nd	nd
16	*Glomus fasciculatum*	nd	nd	3.13	nd	nd	6.34	nd	0.98	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
17	*Glomus microaggregatum*	nd	nd	nd	1.22	0.94	nd	1.96	nd	3.39	nd	1.09	nd	nd	nd	nd	1.68	nd	nd
18	*Glomus multicaule*	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.33	nd	nd	nd
19	*Racocetra gregaria*	nd	nd	nd	nd	1.72	nd	14.38	0.70	nd	1.50	nd	nd	3.73	1.94	9.04	nd	nd	6.09
20	*Rhizoglomus intraradices*	nd	nd	nd	5.28	31.92	nd	nd	1.26	nd	nd	nd	2.29	nd	nd	nd	nd	nd	nd
21	*Scutellospora reticulata*	1.40	nd	5.21	2.03	3.60	nd	9.48	1.96	nd	nd	nd	1.69	9.43	10.03	6.81	1.17	4.44	0.54
22	*Scutellospora scutata*	3.96	nd	nd	10.16	nd	nd	2.94	5.74	3.95	2.25	3.43	nd	5.04	nd	nd	nd	nd	5.02

Legend: nd= **AM species not detected in the sites**; **S-I** = Sangolda-I, **S-II** = Sangolda-II, **S-III** = Sangolda-III, **S-IV** = Macasana, **S-V** = Moira, **S-VI** = Dadachiwadi Dhargalim, **S-VII** = Deulwada Harmal, **S-VIII** = Harmal Pernem, **S-IX** = Corjuem, **S-X** = Coimavaddo Aldona, **S-XI** = Utorda, **S-XII** = Mencurem, **S-XIII** = Ozarim-I, **S-XIV** = Ozarim-II, **S-XV** = Dulape, **S-XVI** = Colva, **S-XVII** = Porvorim, and **S-XVIII** = Guirim.

Table 5.5: Relative abundance (RA), isolation frequency (IF), and frequency of occurrence (FO) of AM fungal species.

Sr. No.	AM fungal species	RA %	IF %	FO
1	*Acaulospora delicata* Walker, Pfeiff. & Bloss	0.79	22.22	12
2	*Acaulospora foveata* Trappe & Janos	3.25	50.00	27
3	*Acaulospora mellea* Spain & Schenck	14.09	83.33	45
4	*Acaulospora myriocarpa* Spain, Sieverd. & Schenck	3.53	33.33	18
5	*Acaulospora polonica* Blaszk.	2.47	33.33	18
6	*Acaulospora scrobiculata* Trappe	0.31	16.67	9
7	*Acaulospora spinosa* Walker & Trappe	0.69	16.67	9
8	*Acaulospora undulata* Sieverd	12.43	94.44	51
9	*Funneliformis geosporum* (Nicolson & Gerd.) Walker & Schüßler	0.24	16.67	9
10	*Funneliformis mosseae* (Nicolson & Gerd.) Walker & Schüßler	0.16	11.11	6
11	*Gigaspora albida* Schenck & Sm.	0.94	27.78	15
12	*Gigaspora decipiens* Hall & Abbott	28.01	100.00	54
13	*Gigaspora gigantea* (Nicolson & Gerd.) Gerd. & Trappe	0.82	16.67	9
14	*Gigaspora margarita* Becker & Hall	19.57	83.33	45
15	*Glomus albidum* Walker & Rhodes	1.23	27.78	15
16	*Glomus fasciculatum* (Thaxter) Walker & Koske	0.38	16.67	9
17	*Glomus microaggregatum* Koske, Gemma & Olexia	0.52	33.33	18
18	*Glomus multicaule* Gerdemann & Bakshi	0.09	5.56	3
19	*Racocetra gregaria* (Schenck & Nicolson) Oehl, Souza & Sieverd.	2.05	44.44	24
20	*Rhizoglomus intraradices* (Schenck & Sm.) Walker & Schüßler	2.84	22.22	12
21	*Scutellospora reticulata* (Koske & Walker), Walker & Sanders	3.00	72.22	39
22	*Scutellospora scutata* C. Walker & Dieder	2.59	50.00	27

Table 5.6: Spearman's rank correlation test between environmental variables and spore density, species richness, species evenness and species diversity.

		EC	pH	OC	N	P	K	Zn	Fe	Mn	Cu	Spore density	Species richness	Species evenness	Species diversity
EC	Correlation Coefficient	.													
	Sig. (2-tailed)														
pH	Correlation Coefficient	0.221													
	Sig. (2-tailed)	0.108	.												
OC	Correlation Coefficient	-.321*	-0.123												
	Sig. (2-tailed)	0.018	0.376	.											
N	Correlation Coefficient	-.378**	-.281*	.398**											
	Sig. (2-tailed)	0.005	0.039	0.003	.										
P	Correlation Coefficient	.682**	.331*	-0.266	-.352**										
	Sig. (2-tailed)	0	0.015	0.052	0.009	.									
K	Correlation Coefficient	-.507**	-.405**	0.227	.306*	-.421**									
	Sig. (2-tailed)	0	0.002	0.099	0.024	0.002	.								
Zn	Correlation Coefficient	0.24	0.116	0.231	-0.099	0.209	-0.254								
	Sig. (2-tailed)	0.08	0.403	0.093	0.478	0.13	0.064	.							
Fe	Correlation Coefficient	0.256	0.114	-0.007	-.341*	.364**	-0.257	-0.077							
	Sig. (2-tailed)	0.062	0.412	0.963	0.012	0.007	0.061	0.58	.						
Mn	Correlation Coefficient	0.205	0.211	.290*	0.032	-0.026	-.479**	.317*	-0.025						
	Sig. (2-tailed)	0.138	0.126	0.034	0.816	0.852	0	0.02	0.857	.					
Cu	Correlation Coefficient	-0.138	-0.195	-0.068	0.243	-0.264	0.142	-0.225	-0.208	-0.167					
	Sig. (2-tailed)	0.321	0.158	0.625	0.077	0.054	0.307	0.102	0.132	0.227	.				
Spore density	Correlation Coefficient	-0.018	.392**	-0.052	-0.256	.390**	-0.21	-0.07	0.108	-0.141	-.364**				
	Sig. (2-tailed)	0.899	0.003	0.711	0.062	0.004	0.127	0.613	0.437	0.31	0.007	.			
Species Richness	Correlation Coefficient	-.293*	0.156	-0.164	-0.143	-0.094	0.183	-0.24	-0.211	-.486**	.517**	0.054			
	Sig. (2-tailed)	0.032	0.259	0.237	0.301	0.498	0.185	0.081	0.125	0	0	0.698			
Species Eveness	Correlation Coefficient	0.182	0.115	-0.242	0.15	-0.011	0.136	-0.219	-0.132	-.344*	0.205	-0.169	0.04		
	Sig. (2-tailed)	0.187	0.409	0.078	0.279	0.935	0.327	0.112	0.34	0.011	0.137	0.222	0.775	.	
Species diversity	Correlation Coefficient	-0.15	0.248	-0.219	-0.096	-0.084	0.22	-.395**	-0.204	-.573**	.362**	0.152	.732**	.627**	
	Sig. (2-tailed)	0.28	0.071	0.111	0.489	0.545	0.11	0.003	0.139	0	0.007	0.272	0	0	.

Legend: EC, **pH**, Organic Carbon **(OC)**, Nitrogen **(N)**, Phosphorus **(P)**, Potassium **(K)**, Zinc **(Zn)**, Iron **(Fe)**, Manganese **(Mn)** and Copper **(Cu)**.

5.4: CONCLUSION

The present study revealed variation in AM fungal diversity in the cowpea-grown agricultural fields. The cowpea legume assists in BNF and thus increases the N fertility of soils. In Goa, cowpea is known to have wide variability in phenotypic and genotypic traits and harness better returns for the farmers. Therefore a systematic survey was carried out to identify superior accession/s with desirable attributes for large-scale cultivation. The current research efforts have identified profitable acquisitions and recorded an appreciable amount of AM fungal diversity in the cowpea crop. However, a detailed study on the AM fungal species, soil fertility, fertilizer application, host plant phenology, and competitive abilities of co-occurring AM fungal species is needed to develop an efficient bio-inoculum for improving crop productivity in cowpea.

CHAPTER 6: Isolation and molecular identification of the Rhizobial strains associated with *Vigna unguiculata*

6.1: INTRODUCTION

Legumes play a significant role in N fixation in an agricultural system with the symbiosis of rhizobia soil bacteria. Cowpea plays an essential role in BNF through legume-rhizobia association (Mohale *et al*., 2013) which potentially provides significant N inputs for agricultural production (Echevarria-Zomeno *et al*., 2009). Studies have also shown that BNF in farmers' fields contributed N up to 66% in Botswana (Jaiswal and Dakora, 2019) and 99% in Ghana (Naab, 2009). In agricultural land without irrigation and agrochemicals inputs limit crop production, and a decline in fertility and agricultural intensification reduces the smallholder farmers' farm size (Giller *et al*., 2015). Singh *et al*., (2003) reported several factors, such as disease, insects, and pests at pre- and post-harvest stages, affecting cowpea production in the tropics. It is found that the influence of rhizobial community structure and function is mainly because of the environmental factors, soil pH, and mineral nutrients (Mohammed *et al*., 2018). Puozaa *et al*., (2019) have shown that the diversity of *Bradyrhizobium* isolates was mainly influenced by N, P, and Na contents in the soil.

In cowpea, nodulation is brought about by both rhizobia and bradyrhizobia. The Genus *Bradyrhizobium* is currently composed of 19 species and is suggested as an ancestor of all rhizobia based on the International Committee of Taxonomy (Parker, 2015). In bacteria, the phylogenetic relationship is assessed based on the 16S rRNA gene, including *Bradyrhizobium* is highly conserved and has limiting species definition (Delamuta *et al*., 2012). In the agricultural system, the population of indigenous rhizobial species is affected by crop history and crop system (Zilli *et al*., 2004). Further, based on the range of effectiveness, the host specificity was observed in cowpea bradyrhizobia (Thies *et al*., 1991). However, in legumes, cross-nodulation by *Bradyrhizobium* is commonly observed. Some of the most important grain legumes of the world include *Vigna unguiculata, V. radiata, Arachis hypogaea, Glycine max,* and *Psophocarpus tetragonolobus,* as well as pasture plants and other tropical covers usually host *Bradyrhizobium* species.

Silva *et al*., (2014) isolated and reported six strains from cowpea nodules and, based on the 16S rRNA gene sequence analysis, a distinct group within the genus *Bradyrhizobium*.

Also, the rhizobial phylogeny was organized into five genera based on the 16S rRNA rhizobia *viz*., *Bradyrhizobium, Azorhizobium, Mesorhizobium, Ensifer (Sinorhizobium),* and *Rhizobium* (Degefu *et al*., 2018). Studies from the subtropical regions of China revealed that *Bradyrhizobium* species which occupied 90% of the cowpea and mung bean nodules and others, were fast-growing rhizobia. A study from Sub-Saharan Africa reported that from two cowpea varieties (IT-1263 and IT-18), 122 micro-symbionts were isolated, and 17 clades were grouped based on BOX-PCR dendrogram (Chidebe *et al*., 2018). In the world's cultivated land, rhizobia reported high molecular diversity (Guimaraes *et al*., 2012).

Soil pH and mineral nutrients influence rhizobial community structure and function (Mohammed *et al*., 2019). Major nutrient inputs, especially N and P, play an essential role in cowpea production. Since the cowpea cultivars are indigenous to Goa, identifying and characterizing their rhizobia will help understand the factors shaping the rhizobial diversity and distribution in the soil. Therefore, this study aimed to isolate and carry out the molecular characterization of rhizobial isolates nodulating cowpea from the different sites undertaken for the study.

6.2: MATERIALS AND METHODS

6.2.1: Isolation of bacteria and preparation of pure cultures

Isolation of rhizobia was carried out using a Yeast Extract Mannitol Agar (YEMA) medium. Healthy, unbroken, firm, and pink nodules were selected to isolate root nodule bacteria (Vincent, 1970). The nodules were removed from the root leaving a small portion of the root attached to each for handling. They were then washed thoroughly in running tap water to remove adhering mud and soil particles and treated or immersed in a 0.1% (w/v) acidified mercuric chloride solution for 4-5 minutes, then agitating in 70% ethanol solution for 3-5 minutes. Finally, nodules were washed thoroughly in sterile distilled water to remove all traces of the disinfectant. The nodules were then crushed in a small aliquot of sterile water in a sterile test tube with the help of a sterile glass rod. The milky exudate thus obtained was inoculated onto Petri plates of YEMA medium containing 1% Congo-red dye and incubated at 28 ± 1^{0}C for 24 hours.

Growth on the YEMA plates was observed after incubation. The well-isolated colonies obtained from the subculture were transferred to YEMA slants containing 0.1% calcium carbonate for maintenance and further studies. The slants were kept at 4^0C in the refrigerator with regular transfers every three months. The cultures were coded according to the botanical name of the host plant from which they were isolated. To confirm that the isolates belong to the genus *Bradyrhizobium* and *Rhizobium* and not *Agrobacterium* or other non-rhizobial contaminants, the bacterial staining, colony characteristics, and nodulation test were performed. After confirming the culture's identity, molecular sequencing was done at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India.

6.2.2: Identification of root nodule bacteria

I) Staining: Gram staining of the rhizobial cultures was determined by the staining method described by Vincent (1970).

II) Colony characteristics: For morphological characterization, the isolated colonies were sub-cultured on YEMA plates and incubated for 2-10 days at 28^0C. The colony morphology was evaluated, and various parameters *viz.,* colour, form and elevation, margins, size, and mucus morphology were recorded.

6.2.3: Nodulation test with isolates

The single colonies of the bacterial isolates were examined for their ability to form root nodules on cowpea, their homologous host plant. Healthy cowpea seeds were surface-sterilized by immersing in 75% alcohol for 10 seconds. The alcohol was drained, and the seeds were placed in 3% sodium hypochlorite for 1-2 minutes. Seeds were then rinsed six times with sterile distilled water, and four seeds were planted in sterile autoclaved sand in sterile pots with three replications under glasshouse conditions. Six-day-old seedlings were inoculated with 1 mL bacterial suspension using sterile micropipettes. Uninoculated plants served as a control. The test seedlings were added with sterile N-free nutrient solution (Broughton and Dilworth, 1971) twice a week and sterile distilled water when necessary. Eight weeks after planting, the plants were harvested to check the nodulation efficiency.

6.2.4: Scanning Electron Microscopy (SEM) preparation for bacterial isolates

Bacteria colonies were selected from a three-day-old culture grown on a YEMA medium. The sample was inserted into a filter disc (pore size 16-40 µm) and placed into the filter discs and porous pots. Smooth mucilaginous biofilm was prepared for SEM study using fixation and dehydration protocols. The most commonly used fixative is Glutaraldehyde (10%) fixation with air drying (two hours in 10% glutaraldehyde in 0.1M potassium phosphate buffer, pH 7.0) (Boyde and Vesely, 1972), six hours under a fume hood and dehydrated in a graded ethanol series 35%, 50%, 70%, 95% and 100% ethanol (1x10 min). After drying, the specimens were mounted on aluminium stubs with carbon tape and then coated with a gold-palladium alloy using a Polaron SC7620 sputter coater. The samples were coated for 30 minutes, resulting in a relatively thin coat that prevented the formation of gold-coating artifacts. The coated specimen was examined in an SEM, Carl ZEISS EVO 18 (Folk and Lynch, 1997).

6.2.5: Molecular identification of the bacterial strains

6.2.5.1: Genomic DNA Isolation from bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. A part of the culture was taken in a microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K were added and incubated at 56^0C in a water bath until completely lysed. After lysis, 5 µl of RNase A (100 mg mL^{-1}) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70^0C for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into a NucleoSpin® Tissue column, placed in a 2 mL collection tube, and centrifuged at 11000x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 mL tube and washed with 500 µl of BW buffer. The Wash step was repeated using 600 µl of B5 buffer. After washing, the NucleoSpin® Tissue column was placed in a clean 1.5 mL tube, and DNA was eluted out using 50 µl of BE buffer.

6.2.5.2: Amplification of PCR 16S rRNA gene

The 16S rRNA genes from the bacterial cultures were amplified by Polymerase Chain Reaction (PCR) using 16S-RS-F (CAGGCCTAACACATGCAAGTC) -16S-RS-R

(GGGCGGWGTGTACAAGGC) primer-pair. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

The PCR amplification was carried out with the following components:

Reaction mixture	**Quantity (µL)**
2X Phire Master Mix	5.0
D/W	4.0
Forward Primer	0.25
Reverse Primer	0.25
DNA	1.0

The following temperature transitions were used:

Temperature (^{0}C)	**Time**
95	5 min
95	30 sec
60	40 sec
72	60 sec
72	7 min
4	∞

The thermal cycler was programmed for 35 cycles, with one cycle for the first step of denaturation and 35 cycles for steps 2-4.

6.2.5.3: Agarose Gel electrophoresis of PCR products

The PCR products were resolved by electrophoresis using 1.2% agarose gels prepared in 0.5X TBE buffer stained with 0.5 µg mL^{-1} ethidium bromide. 1 µl of 6X loading dye was mixed with 4 µl of PCR products and was loaded. A 2-log DNA ladder (NEB) was used as a marker, and electrophoresis was performed at a 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours. The gel was visualized in a UV transilluminator (Genei), and the image was captured under UV light using a Gel documentation system (Bio-Rad).

6.2.5.4: ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), which were used to remove unwanted primers and dNTPs from a PCR product. A 5 µL of PCR product was mixed with 0.5µl of ExoSAP-IT and incubated at 37^0C for 15 minutes, followed by enzyme inactivation at 85^0C for 5 minutes.

6.2.5.5: DNA sequencing

The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

The Sequencing PCR mix consisted of the following components:

Reaction mixture	**Quantity (µL)**
D/W	6.6
5X Sequencing Buffer	1.9
Forward Primer	0.3
Reverse Primer	0.3
Sequencing Mix	0.2
ExoSAP treated PCR product	1.0

Sequencing PCR amplification profile

Temperature (^{0}C)	**Time**
96	2 min
96	30 sec
50	40 sec
60	4 min
40	∞

6.2.5.6: Sequence alignment and phylogenetic analysis

The sequences were blasted in GenBank with Blasts, and further related sequences were assembled. A phylogenetic analysis was conducted using maximum likelihood (ML) in MEGA X (Kumar *et al*., 2018) with 1,000 bootstrap replicates. The most suitable substitution model was selected using the MEG X Tamura Nei model was used in the analysis. Gaps were treated as a pair-wise deletion, and the tree was viewed with Mega X. All newly generated sequences used in this study are deposited in GenBank.

6.3: RESULTS AND DISCUSSION

6.3.1: Isolated rhizobial strains and their morphological characteristics

Ten isolates were obtained from the cowpea nodules collected from all the 18 study sites in Goa. The effective rhizobial isolates differed significantly in morphological characteristics, with colony texture showing the isolates as gummy, dry, and elastic. About 94% of the rhizobial isolates appeared in 2-4 days, and 6% took 7-10 days to appear on YEMA plates. Colony shapes varied and were observed irregular, domed, conical, watery, circular, and flat. Colony colour differed with whitish, milky, and translucent on YEMA plates. Two rhizobial strains, *viz.*, GUBRS1 and GUBRS2, were isolated based on colony characteristics. Microscopic observation showed that the cells of isolates were motile, gram-negative, tiny rod-shaped structures **(Plate 6.1).** The microscopic identification of the rhizobial species was conducted using the Scanning Electron Microscope (SEM) technique (**Plate 6.2).** In an earlier study, Gaafar (2020) carried out the taxonomic identification of the legume surface in seven species of *Vigna* (11 accessions) based on the SEM technique.

The present study results confirmed that the *Bradyrhizobium* strains isolated from the *V. unguiculata* were responsible for nodulation. They were fast-growing and were successfully cultured on the YEMA medium. Gronemeyer *et al*., (2014) reported cowpea nodulation by both *Bradyrhizobium* and *Rhizobium* species. The study shows that nodule-forming rhizobia only took 2-4 days to grow on YEMA plates and was considered a fast-growing rhizobial species. At the same time, Chidebe *et al*., (2018) reported slow-growing (*Bradyrhizobium*) and fast-growing (*Rhizobium*) species in cowpea.

6.3.2: Nodulation test with isolates

The rhizobial isolates were tested in a pot experiment using sterilized sand culture to assess their infectivity and effectiveness on cowpea under glasshouse conditions. The results confirmed that the strains on cowpea formed nodules and were true rhizobia infecting their host upon reinoculation. A similar study, Ayele (2010) obtained a 100% infection and nodule formation upon reinoculation of *Bradyrhizobium* isolates on groundnut in eastern Ethiopia. Further, the appearance of dark green leaf colour and pink nodules was considered an indication of effective nodulation. Only effective nodules forming isolates were used for further studies **(Plate 6.3).**

6.3.3: Molecular identification of the isolates

6.3.3.1: Phylogenetic analysis

The *in vitro* bacterial isolate selected based on its ability to improve the growth of cowpea plants was identified by multilocus sequence analysis as a species of *Bradyrhizobium*. It is essential to identify microsymbionts taxonomy and phylogeny to improve legume production using BNF (Chidebe *et al*., 2018). Twenty-three taxa were included in the phylogenetic analysis **(Table 6.1; Fig. 6.1).** The result of the phylogenetic analysis showed that the strains under consideration formed a distinct lineage within the genus *Bradyrhizobium. Rhizobium lusitanum* was selected as the outgroup taxon. The PCR amplification of the 16S rRNA gene yielded ~1.5 kb amplicons of full length for the test isolates. The BLAST 16S rRNA sequence analysis of these isolates showed that all the strains had high sequence similarities with *Bradyrhizobium* with 100% conserved and variable sequence sites.

The maximum likelihood phylogeny of the 16S rRNA gene placed the test isolates into distinct cluster isolates, GUBRS1 and GUBRS2 shared 100% sequence identify with *B. japonicum* Hpa GG5 and *B. japonicum* L16. Obtained sequences were deposited in the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers MH675487 and MH675491. A similar study from Brazil wherein BR3267 and BR3262 strains were characterized based on 16S rRNA genes and concluded that both rhizobia belong to *Bradyrhizobium* (Zilli and Valicheski, 2006). In our study, analysis with $BLAST_n$ sequence of 16S rRNA alignment showed *Bradyrhizobium* species. Wherein, Chidbe *et al.,* (2018) showed that $BLAST_n$ sequence analysis of 16S rRNA and four

housekeeping genes (gyrB, glnII, rpoB, and recA) showed their alignment with *Rhizobium* and *Bradyrhizobium* species.

Table 6.1: Origin of DNA sequences and newly deposited sequences (in bold).

Sr. No.	Taxon	Genebank number
1.	*Bradyrhizobium arachidis* strain 65078	MN661167
2.	*Bradyrhizobium liaoningense* strain 62318	MN661168
3.	*Bradyrhizobium liaoningense* strain 60954	MN661164
4.	*Bradyrhizobium liaoningense* strain NAC66	MK872349
5.	*Bradyrhizobium liaoningense* strain PZHK1	KU530191
6.	*Bradyrhizobium daqingense* strain IND-10A	KX230052
7.	*Bradyrhizobium americanum* strain CMVU44	NR149803
8.	*Bradyrhizobium shewense* RP6	LR130151
9.	*Bradyrhizobium liaoningense* strain NAC63	MK872347
10.	*Bradyrhizobium lupini*	MN525211
11.	*Bradyrhizobium japonicum* strain L16	KY412844
12.	*Bradyrhizobium japonicum* HpaGG5	LC515505
13.	*Bradyrhizobium diazoefficiens* USDA 110	AB909430
14.	*Bradyrhizobium rifense* strain CTAW71	NR149804
15.	*Bradyrhizobium arachidis* strain CCBAU 051107	HM107167
16.	*Bradyrhizobium embrapense* strain SEMIA 6208	NR145861
17.	*Bradyrhizobium tropiciagri* strain SEMIA 6148	NR145862
18.	*Bradyrhizobium jicamae* strain PAC68	AY624134
19.	*Bradyrhizobium lablabi* strain CCBAU 23086	GU433448
20.	*Bradyrhizobium erythrophlei* strain CCBAU 53325	NR135877
21.	*Rhizobium lusitanum* strain P1-7	NR043150
22.	***Bradyrhizobium* sp. GUBRS1**	**MH675487**
23.	***Bradyrhizobium* sp. GUBRS2**	**MH675491**

Dabo *et al*., (2019) reported the usefulness of genes for phylogenetic tree construction of the genus *Bradyrhizobium*, such as at PD, gyrB, gInII, symbiotic nifH, and nod C. These housekeeping genes were similar to the 16S rRNA gene tree. The 16S rRNA gene

sequence analysis helps establish the phylogenies of legume microsymbionts (Zinga *et al.*, 2017; Puozaa *et al.*, 2017). Also, the 16S rRNA gene is used in multilocus sequence analysis in combination due to the lower resolving power of the gene (Jaiswal *et al.*, 2016; Tampakaki *et al.*, 2017).

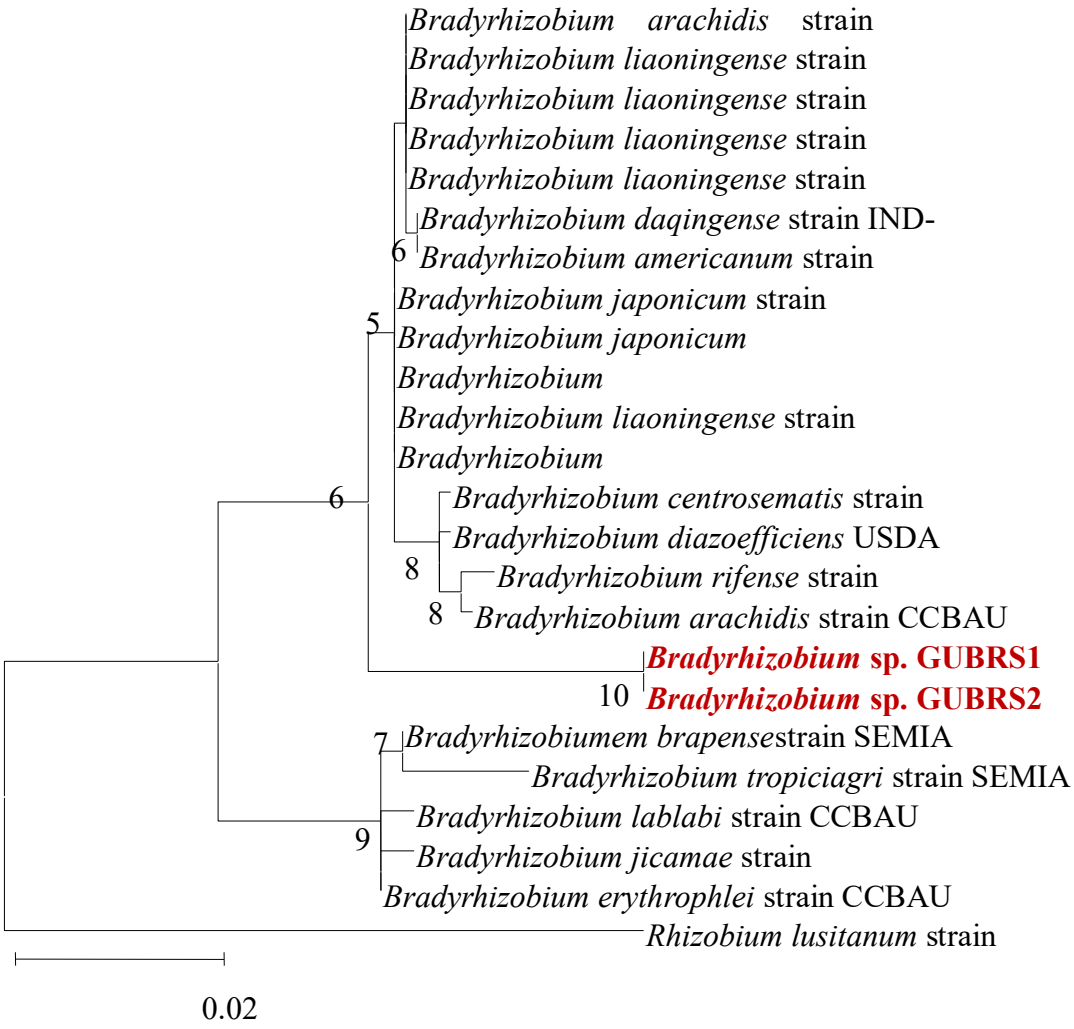


Fig 6.1: Molecular identification of the GUBRS1 and GUBRS2 isolates. The phylogenetic relationship of *Bradyrhizobium* species GUBRS1 and GUBRS2 is based on the sequences of the 16S rRNA gene (1202 bp) and closely related sequences obtained from GenBank. Maximum likelihood tree of *Bradyrhizobium* and related species based on analysis of 16S RNA sequences. The species described in this study are tagged in red colour.

A novel *Bradyrhizobium* species *viz.*, GUBRS2, responsible for cowpea nodulation was identified in this study. Several studies have reported *Bradyrhizobium* symbionts isolated

from cowpea nodulation (Degefu *et al*., 2017; Marinho *et al*., 2017; Chidebe *et al*., 2018; Mohammed *et al*., 2018; Jaiswal and Dakora, 2019; Puozaa *et al*., 2019). Molecular diversity among 122 microsymbionts isolated from Mozambican soil reported diverse cowpea nodulating rhizobia, *viz., Bradyrhizobium arachidis, B. pachyrhizi, B. myuanmingense,* and a novel *Bradyrhizobium* sp., besides *Rhizobium pusense, Rhizobium tropici,* and *Neorhizobium galegae* (Chidbe *et al.,* 2018).

Moreover, several studies have also reported cowpea rhizobia nodulation with different species from the genera *Bradyrhizobium, Rhizobium, Achromobacter, Sinorhizobium, Microvirga,* and *Ralstonia* (Guimaraes *et at*., 2012; Radl *et al.,* 2014; Bejarano *et al*., 2014). To fulfill the growing population's food demand, it is vital to increase agricultural productivity in a sustainable and environmental pollution-free manner. The farmer uses a high cost of inorganic fertilizers to increase crop yield, which pollutes the environment (Jaiswal and Dakora, 2019) and is costly for poor farmers (Chianu *et al.,* 2011). However, in legume crops through BNF, N demand can provide farmers with economic, environmental, and agroeconomic benefits (Siddique *et al*., 2012). The present study showed that the isolated indigenous rhizobial symbionts exhibited nodulation in cowpea, demonstrating their potential for use in inoculants formulation to achieve maximum legume productivity. As an alternative to chemical-based fertilizers, highly effective indigenous rhizobial symbionts are most important for supplying sufficient N for maximum legume production (Odori *et al*., 2020).

Soil Physico-chemical properties, as described in Chapter 4, play an essential role in influencing the diversity of *Bradyrhizobium* species. This finding is well supported by earlier studies (Rathi *et al*., 2018; Ojha *et al*., 2017; Puozaa *et al*., 2017). The present study reports the acidic nature of the soils at the various study sites. Studies suggest that some species tolerate acidic to alkaline soil conditions for their origin (Bünger *et al*., 2018; Ahnia *et al*., 2018). In cowpea grown soils, *Bradyrhizobium* species appear to be predominant microsymbionts and play a key role in overcoming P and N nutrients (de Souza *et al*., 2006).

6.4: CONCLUSION

The two rhizobial strains isolated from cowpea were sequenced and analyzed for molecular identification using the NCBI BLASTn tool based on the sequence match with

an earlier reported sequence of type strains. The results revealed sequence similarity with the genus *Bradyrhizobium*. It is also observed that irrespective of soil type and plant genotype, *Bradyrhizobium* species were the significant symbionts of cowpea. Also, in the present study, the soil pH varied from 4.7 to 5.9. This may be due to fertilization and higher precipitation in the soils due to Al, Mn, and Fe solubility.

Rhizobial bacteria were tolerant to acidic soil and could effectively nodulate cowpea. It is suggested that the fast-growing strains are candidates for acidic soils and also could be employed as commercial inoculants based on adaptation to extreme conditions. Therefore, in cowpea agricultural soils, *Bradyrhizobium* appears as the predominant microsymbiont that plays a crucial role in N nutrition. The present study suggests that acidic soil and edaphic factors are responsible for the diversity of indigenous and novel *Bradyrhizobium* species. The results confirmed that the isolated indigenous rhizobial symbionts exhibited nodulation in cowpea, demonstrating their potential for use in inoculants formulation to achieve maximum legume productivity.

CHAPTER 7: Standardization of mass multiplication of AM species using pot cultures and *in-vitro* techniques

7.1: INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are eukaryotic, multinucleate, asexually reproducing obligate biotrophs. They have a predominant ancestral association with land plants and are known for their extensive hyphal growth in the soil (Simard and Austin, 2010). These fungi are estimated to be present in 93% of flowering plants and 92% of land plant families (File *et al*., 2012). AM fungi play a vital role in plant communities' structure and retrieval of degraded regions with plant vegetation, apart from the numerous benefits. They are of agricultural and horticultural importance (Ijdo *et al*., 2011). AM fungi play a significant role in improving soil characteristics and enhancing above-ground and below-ground biodiversity. They aid the plant to survive and grow even under nutrient- or moisture-stress conditions and protect the plant from infection under disturbed soil conditions (Brundrett and Abbott, 2002).

There is, however, a practical difficulty in their application. Field-collected spores are usually low in number and sometimes parasitized or deteriorated. This leads to a lack of taxonomic information. Further, spores recovered from the field even though healthy and infective due to changes in environmental conditions, soil type, and organic matter content vary in colonization levels. Also, spores may be grazed by insects or colonized by microbes. As a result, they may not be viable.

Moreover, all the AM fungal species may not sporulate in field conditions. Several of these difficulties can be overcome, and maximum AM fungal species can be obtained in the collection by using trap and pot culture. Trap culture is vital for getting efficient results for AM fungal inoculations. It is used to isolate inherent AM fungi and introduce monospecific inoculum. The technique depends on biotic and abiotic factors, growth, colonization, and sporulation of AM fungi. The AM fungi obtained in trap cultures are further maintained through pot cultures.

AM fungal indigenous diversity can be accessed widely through trap cultures. In this culture system, the host plant plays an important role. Trap cultures help obtain healthy spores of colonizing fungi for monospecific culture preparation for subsequent pot

cultures. Trap culture can be established using rhizosphere soil mixture, root pieces, and bulk soil with sterilized sand grown with suitable hosts. Usually, trap cultures uncover many newly established, healthy spores that help assess AM species diversity in different ecosystems. However, mysterious species from the field sampling are known to appear in trap cultures (Stürmer, 2004). A study by Miller *et al*., (1985) showed that when the apple orchard soil sample was subjected to sorghum and coleus trap culture, 14 AM fungal species were recovered, those previously not reported from the apple orchard fields.

The pot culture technique is used to culture, isolate, and maintain AM fungi (Gilmore, 1968). AM fungi are propagated based on host plants for good colonization and sporulation in the pot culture method. Spores cultured in pot culture are uniform size, growth period, and distinct morphology. Isolated, contamination-free pot cultures protect the fungal propagules from predators such as arthropods, rodents, insects, and pests.

Pot culture with sterilized soil or through hydroponics, aeroponics, or other greenhouse methods has been used in large-scale AM fungal production (Ijdo *et al*., 2011). Millner and Kitt (1992) introduced an automated water system in an open pot culture to overcome maintenance costs. A single host plant species may affect the mycorrhization based on soil water, pH, salinity, temperature, P level, and light intensity. However, the host plant should be obligatorily mycotrophic. It should have good tolerance, extensive root growth, high photosynthetic efficiency, phosphate acquisition capability, plant-pathogen resistance, and support sporulation of all AM fungal species.

The most widely used standard and conventional method of maintaining pot cultures are mixing soil, spores, hyphal segments, and root pieces colonized by AM fungal inoculum. For inoculum production, sterilized soil mixture, peat, perlite, and vermiculite are used as substrates (Millner and Kitt, 1992; Douds *et al*., 2006; Plenchette *et al*., 1983). The pH plays a vital role in a successful pure culture establishment (Mosse and Hepper, 1975). Besides, geographical location, habitat, edaphic factors, host identity, and environmental heterogeneity also have a role to play. A significant drawback of using pot cultures, as reported on the INVAM website (http://invam.caf.wvu.edu), is that spore numbers decrease in some pot cultures even after successive propagation cycles.

Among the newer AM fungal culture maintenance methods is an *in vitro* monoxenic culture technique that uses root organ culture (Tiwari and Adoleya, 2002; Ijdo *et al*., 2011). AM fungi have been successfully propagated on *Agrobacterium rhizogenes* transformed roots (Becard and Fotin, 1988; Adholeya *et al*., 2005; Schultze, 2013). The technique uses Modified Strulla-Romand (MSR) and Minimal mineral media (M) for *in vitro* culture (Bago *et al*., 1996). The use of hairy carrot root as a host organ is the most widespread (Becard and Fortin, 1988). However, endomycorrhizal colonization and sporulation on the transformed root of *Linum usitatissimum* L. (Linum) have also been established recently (Rodrigues and Rodrigues, 2015).

Inoculum production using monoxenic cultivation has several advantages over conventional cultivation systems. While open cultures of AM fungi are inclined to contamination with other AM fungi, the monoxenic culture technique prevents contamination (Mosse and Hepper, 1975). Further, this technique provides scope for large-scale production of AM fungal inoculum and a good amount of extra-radical spore within a short period (Adholeya *et al*., 2005; Bidondo *et al*., 2012). In the agricultural system, monoxenically produced contaminant-free AM inoculum is valuable. However, *in vitro* monoxenic cultures have their drawbacks and are not widely practiced. The disadvantages include unavoidable contamination, skilled personnel, demanding technology and identification of each genotype, and culturing limits (Bago and Cano, 2005; Fortin *et al*., 2005). Minimizing the drawbacks will help produce large bio-inoculants, further reducing dependence on chemical fertilizers. It will be a promising step towards sustainable agriculture, particularly for marginal farming communities. The present study is aimed to establish an *in vitro* culture technique for mass multiplication of indigenous AM fungal cultures isolated from rhizosphere soils of cowpea-grown agricultural soils.

7.2: MATERIALS AND METHODS

7.2.1: Trap culture preparation

AM fungal community was propagated using the modified trap culture method (Morton *et al*., 1993). Trap culture was prepared by mixing sand (sterilized at 90^0C for 72 h and cooled to room temperature and soil inoculum (rhizosphere soil and roots) in a ratio of 1:1. The mixture was then transferred to surface sterilized (wiped with absolute alcohol) plastic pots. *Plectranthus scutellarioides* (L.) R.Br. (Lamiaceae), popularly known as the coleus,

was used as a catch plant. For this purpose, coleus was grown separately from cuttings in sterilized sand before transplanting. The plant cuttings were surface-sterilized, sown directly into the pot, and maintained under polyhouse at 27^0C. The pots were watered once every two days. After 90 days, the pots were left to dry undisturbed (1-2 weeks), following which the shoot portion was cut off at the soil interface. The soil was separated from the roots. The roots were washed free of soil and were transferred in *Ziploc* polythene bags, labeled, and stored at 4^0C for at least 30 days to eliminate or reduce the dormancy period. AM fungal spores were recovered for identification purposes from the soil, and roots were used to determine mycorrhizal colonization. The extracted spores were also used to prepare monospecific cultures.

7.2.2: Preparation of Monospecific (single species) cultures

Spores were isolated from trap culture using the wet sieving and decanting method (Gerdemann and Nicolson, 1963), as described in Chapter 3. Monospecific cultures were prepared according to the procedure given by Gilmore (1968). Isolated single spores of AM fungi were washed repeatedly and stored in Petri plates at 4°C as per the procedure mentioned in https://invam.wvu.edu/methods/culture-methods/single-species-cultures. The isolated spores were examined daily by viewing under a stereomicroscope (Olympus SZ2-ILST) at 45x magnification for any changes in morphology, *i.e.*, loss of spore content, colour change, damage, and parasitism. Such defective spores were excluded. The healthy spores were washed with distilled water to remove any soil particles or hyphal fragments before inoculation in the pot. Plastic pots were wiped with absolute alcohol and filled with sterilized sand. The isolated spores (belonging to single species) were placed with Whatman filter paper at a depth of 2-3 cm in the pots, covered with additional medium, and planted with *P. scutellarioides* cuttings.

The pots were maintained in the glasshouse at 27^0C, with 63% relative humidity. They were watered once every two days, and Hoagland's nutrient (Hoagland and Arnon, 1950) deficient of P was added fortnightly to the pots. After 90 days of the growth period, the pots were stopped watering and drying. The pot content was then analyzed, and spores were recovered. Microscopic observations were carried out using bright-field microscopes (Olympus BX 41 and Nikon Eclipse E200). Photomicrographs were captured using Olympus DP 12-2 and Nikon Digital Sight DS-U3 digital cameras and were not digitally

edited. The isolated spore from monospecific cultures was used for *in vitro* root organ culture technique.

7.2.3: *In vitro* culture of AM fungi using Ri T-DNA transformed roots

7.2.3.1: Extraction of AM fungal propagules

AM fungal propagules were extracted from rhizosphere soil samples and monospecific cultures using the wet sieving and decanting technique (Gerdemann and Nicolson, 1963) as described in Chapter 3.

7.2.3.2: AM fungal propagule disinfection process

For monoxenic culture establishment, isolated propagules were first rinsed twice with sterilized distilled water, after which they were disinfected. A modified method of Mosse (1959) and Becard and Fortin (1988) was employed wherein a combination of 0.5% NaClO (sodium hypochlorite) + 2% chloramine T (3 min) was used. The concentrations and sterilization time varied from species to species (based on the size of spores) and depended on the type of propagules used (spores or colonized root fragments). An amount of 200-400 µl NaClO for 3-5 minutes was optimum. The propagules were rinsed three times with sterilized distilled water and treated with an antibiotic solution (0.02% Streptomycin) for 10 min.

7.2.3.3: Germination of disinfected propagules

Spores/colonized roots of six AM fungal species were used for germination. For each species, 50 disinfected propagules (5 spores or colonized roots/Petri plate x 10 Petri plates) were plated in triplicate on Modified Strullu-Romand (MSR) minus sucrose medium. The Petri plates were incubated in an inverted position in the dark at 27°C. The medium's pH, sucrose, and nutrient composition were varied to obtain the maximum germination percentage. Of the different pH levels (4.8-6.5) tested, pH 6.5 was found suitable for a few AM species. The sucrose content was reduced to half the strength as described by Declerck *et al*., (1998) and was ideal for all AM species tested.

7.2.3.4: Culture medium for cultivation of AM propagules and transformed roots

MSR medium was used to culture AM propagules and transformed roots (Declerck *et al*., 1998).

7.2.3.5: Establishment of Monoxenic culture

Actively growing Ri T-DNA transformed roots of Chicory *(Cichorium intybus* L.) and *Linum* (*Linum usitatissimum* L.) in association with AM germinated propagules was used for the establishment of dual culture in MSR medium. The Petri plates were incubated in an inverted position at 27^0C in the dark. The transformed roots were procured from Prof. B. F. Rodrigues, Department of Botany, Goa University.

7.2.3.6: Extraction of monoxenically produced AM propagules

The monoxenically produced AM propagules were extracted from the inoculated MSR medium using the method proposed by Cranenbrouck *et al*., (2005). A small gel containing extra-radical mycelia and spore (monoxenically produced) was transferred into an empty sterile Petri plate. 25 mL citrate buffer (0.01 M) was filtered through a sterile 0.22 µm syringe-driven MF Millipore Membrane Filter Unit (Millex ®- GS) and added to the Petri plate. The Petri plate was then agitated slowly to enable the gelling agent to dissolve. The spores attached to extra-radical mycelium were then transferred to a new sterile Petri plate containing sterile distilled water using a micropipette by viewing under Olympus stereo microscope SZ2-ILST (45x magnification). The entire procedure was carried out in laminar airflow.

7.2.3.7: Establishment of continuous cultures

The extracted monoxenically produced AM propagules were re-associated with the transformed roots onto fresh MSR media to get continuous cultures (Chabot *et al*., 1992).

7.3: RESULTS AND DISCUSSION

7.3.1: Trap cultures and monospecific cultures

A total of 22 AM fungal species were recovered from selected study sites, of which five monospecific cultures *viz., Gigaspora decipiens, Gi. albida, Racocetra gregaria, Scutellospora scutata,* and *Acaulospora myriocarpa* were successfully mass multiplied using *P. scutellarioides* as a host plant. Maintenance of trap and monospecific cultures is inexpensive and requires low technical support. Therefore, they can be widely adopted for AM spore and inocula production (**Plate 7.1**). Besides, trap cultures also help retain viability and minimize the loss of freshly collected samples (Brundrett *et al.*, 1999). As Walker and Vestberg (1994) suggested, cross-contamination within and between AM

fungal species can be avoided by keeping the monospecific cultures isolated in sun bags. Further, in this study, monospecific cultures were mass multiplied (minimum of 5 pots each), and live cultures were maintained in the polyhouse at Goa University Arbuscular Mycorrhizal Culture Collection (GUAMCC), Goa University, Goa (**Plate 7.2; Plate 7.3**). The cultures were consequently used for further studies.

7.3.2: *In vitro* germination

Literature reports that different AM fungal species can be successfully established through monoxenic cultures (Fortin *et al*., 2002). Many of these studies are focused on understanding spore germination and the symbiotic associations. In the present *in vitro* study, monoxenic culture of 4 AM fungal species *viz., Gigaspora decipiens, Racocetra gregaria, Scutellospora scutata,* and *Rhizoglomus intraradices* using Ri T-DNA transformed *Linum (Linum usitatissimum* L.) roots were cultured on MSR media (**Plate 7.4**).

The days for *in vitro* spore germination varied from species to species, with a minimum in *Gi. decipiens* and maximum in *Rh. intraradices*. The results in the present study indicate that on a modified MSR medium at pH 6.5, *Gi. decipiens* takes three days for germination, while *R. gregaria* and *S. scutata* take 4-6 days through the germination shield (**Table 7.1; Plate 7.5**). These results were based on observations recorded for 50 spores of each AM species. Further, in *Gigaspora* and *Scutellospora* species, the germination and hyphal growth rates varied *in vitro*.

Table 7.1: *In vitro* spore germination in different AM species.

Sr. No.	AM fungal species	Number of days required for germination*
1	*Gigaspora decipiens*	3
2	*Racocetra gregaria*	4-6
3	*Scutellospora scutata*	5-6
4	*Rhizoglomus intraradices*	8-9

*Number of spores observed= 50/ species.

7.3.3: Monoxenic cultures

In the present study, isolated spores, as AM propagules, were extensively used to initiate and establish monoxenic culture. Of the four species that germinated *in vitro,* only one species *viz*., *Gi. decipiens* successfully colonized and sporulated (**Plate 7.6; Plate 7.7; Plate 7.8**). Although the spores of *Rhizoglomus intraradices* recorded germination, no further growth was observed. In *R. gregaria* and *S. scutata, in vitro* root colonization and formation of auxiliary cells were observed, but no sporulation was seen even after repeated subculture (**Plate 7.9; Plate 7.10**). This may be due to *in vitro* conditions, the long vegetative phase of 2-3 months, and the lengthy sporulation process in *Scutellospora* species (Kandula *et al*., 2006). There is no vesicle formation in Gigasporaceae but instead is known to produce auxiliary cells on extra-radical hyphae. Also, Gigasporaceae members contain a high amount of lipids in their auxiliary cells, which function as transitory storage (Pearson and Schweiger, 1993) in reproduction, and as vestiges of relict reproduction structure (Morton and Benny, 1990). Auxiliary cells have also been reported as carbon reserves for spore production and sporulation (de Souza and Declerck, 2003).

Further, *Gi. decipiens* shows auxiliary cells with spike-like globular projections (**Plate 7.7**). Karandashov *et al*., (1999) and Declerck *et al*., (2004) described the pattern of auxiliary cells in *Gi. margarita*, and *S. reticulata*, respectively. In *R. gregaria*, auxiliary cells in aggregates are sub-globose to ovoid (**Plate 7.9**). Similarly, in *S. scutata* the auxiliary cells are found in aggregates (**Plate 7.11**). In the present study, to initiate *in vitro* colonization and sporulation, a modified split plate technique (St-Arnaud *et al*., 1996; Raj *et al*., 2017) on MSR medium was employed. It involves the inoculation of both spore and transformed roots in the same Petri plate in regions containing MSR medium without and with sucrose, respectively. This technique minimizes the effect of relocation of germinating spores, thereby speeding root colonization.

Monoxenic cultures produce high-density AM spore inoculum (Chabot *et al*., 1992; Fortin *et al*., 2002), particularly for *Gigaspora* species *viz*., *Gi. margarita, Gi. roseae* and *Gi. gigantea* (Becard and Piche, 1992; Diop *et al*., 1992). It has been reported that spores of Gigasporaceae members are most effective as propagules compared to Glomeraceae species (Brundrett *et al*., 1999). Also, it has been observed that in *Gigaspora* and *Scutellospora* members, successful *in vitro* colonization occurred using spore propagules compared to mycorrhizal root fragments and extra-radical hyphae (Klironomos and Hart,

2002). On the other hand, Mycorrhizal root fragments are considered effective propagules for *in vitro* culturing *Glomus* and *Acaulospora* species (Biermann and Linderman, 1983).

In the present study, *in vitro* spore germination of *Gi. decipiens* occurred from the germ tube initials of the germination shield. A similar growth pattern was also observed in Giovannetti *et al*., (2010) study. Multiple germ tube formation in *Gi. decipiens* probably could be due to genetic information, a high amount of nuclei close to the spore wall (Maia and Yano-Melo, 2001; de Souza *et al*., 2005), or benefit compatibility with the host (Maia *et al*., 2010). In *Gi. decipiens,* the highest spore production occurred at pH (6.5), whereas *G. clarum* was at pH 4.0 (Costa *et al*., 2013). Similarly, Gigasporaceae members *viz*., *Gigaspora gigantea,* germinate one day after incubation (Koske, 1981), *Gi. margarita* germinated after 72 hours on water agar (Sward, 1981). However, *Scutellospora fulgida* and *S. persica* germinated for two weeks at 24^0C in the dark and showed the formation of mycelial growth and auxiliary cells.

The germ tubes grew and branched in the direction of the transformed roots. The hyphal branching in the culture medium exhibited two growth patterns, *viz.,* apical and lateral. The lateral branches showed the presence of septa. The hyphae successfully colonized the transformed *Linum* roots after 45 days of inoculation and showed dense hyphal colonization. In the present *in vitro* study, in *Gi. decipiens,* a single spore was formed in an intercalary position on sporogenous hyphae. According to Costa *et al*., (2013), *Gi. decipiens* spore formation can occur alone or in a group, in terminal or intercalary positions on sporogenous hyphae. Further, mycelium production from the germinated spore is highly prolific. Although there is a rapid occurrence of auxiliary cells, spore formation takes several weeks (Fortin *et al*., 2002).

In the present study *Gi. decipens, R. gregaria,* and *S. scutata* show typical colony morphology, *i.e.,* mycelium with runner hyphae (RH), branched hyphae (BH), arbuscule-like structure (ALS), or branched absorbing structure (BAS) (**Plate 7.7; Plate 7.9; Plate 7.10**). These structures have been reported earlier by Mosse and Hepper (1975), Becard and Fortin (1988), and Costa *et al*., (2013) in similar studies. Studies have suggested that these structures, particularly the BAS, increase the contact surface of the fungus with the culture medium, thereby improving its nutrient absorption rate (Bago *et al*., 1998a).

An increased number of BAS by a particular strain suggests its ability to absorb the nutrients better. After that, its enhanced delivery to its co-symbiont, the plant, thereby leading to the improved plant growth rate. Further, it is reported that ALS/BAS are unique sites for nutrient acquisition for spore development (Bago *et al*., 1998a). Besides the above structures, extensive hyphal density network and hyphal ramification were observed (Declerck *et al*., 2005). Also, the extra-radical mycelium showed septa formation on the root surface due to cytoplasmic restriction (Bago *et al*., 1998b; Srinivasan and Govindasamy, 2014).

7.3.4: Continuous cultures

In *Gi. decipiens, S. scutata*, and *R. gregaria,* the colonized transformed root fragments were subcultured with extra-radical hyphae from the monoxenic starter culture. In all these, good re-growth was observed within two week time interval. However, with intra-radical structures, regrowth was sometimes challenging to achieve. Similar observations were recorded by Strullu and Romand (1986) and Fortin *et al*., (2002).

In vitro cultivation methods reduce the contamination risks and meet demands for quality mass production on a commercial scale (Ijdo *et al*., 2011). In general criteria, AM fungi should complete their life cycle with good sporulation and fungal-continuity *in vitro* culture conditions (Declerck *et al*., 2005). This would make the monoxenic culture technique a contamination-free, promising, rapid, and high spore-producing method. AM root-organ culture technique has essential implications for producing AM inocula for research and commercial purposes.

In monoxenic culture, *Gi. decipiens* were observed to sporulate after 50-55 days of growth (**Plate 7.7**). This is a significant step in monoxenic culture of AM fungi as it is essential to scale up AM fungal inoculum production (Declerck *et al*., 2001; Ijdo *et al*., 2011). The ability of *Gi. decipiens* strain to sporulate within two months of inoculation is a highly promising step towards considering it for potential inoculum production. Owing to its high BAS formation, high colonization intensity, sporulation, and faster re-growth *Gi. decipiens* can be regarded as an indigenous, stable, and homogenous monoxenic culture species. The species can be further used for mass multiplication and screening studies as biofertilizers.

7.4: CONCLUSION

To study AM fungi's life cycle and the large-scale production of microbiologically high-quality clean inoculum, the *in vitro* cultivation of AM fungi is essential. The monoxenic culture of AM fungi, the long-term experimental study, and detailed observations were successfully established for effective and efficient inoculum production. In the present study, the monoxenic culture of *Gi. decipiens* was successfully established in the transformed hairy roots of *Linum* as a host partner on MSR medium. Further, the culture produced BAS and spores, which are highly desirable characters for considering an AM fungal special for establishing mass production of pure and viable inoculum. The monoxenic method over a short period and in limited space resulted in extensive spore production. Therefore, the indigenous species of *Gi. decipiens* significantly facilitate monoxenic inocula for bioinoculum production.

CHAPTER 8: Formulation of a standard carrier for inoculum (both AM and *Bradyrhizobium*)

8.1: INTRODUCTION

Being obligate biotrophs, AM fungi form symbiotic associations in more than 90% of the flowering plants, ferns, and bryophytes (Ahanger *et al*., 2014). During the early growth of plants, P is known to limit root development. AM fungi play a vital role in P acquisition (Castillo *et al*., 2016). The benefit of this symbiosis include macro-and micro-nutrients uptake, soil aggregate stability, water absorption, drought, and salinity stress suppression, trace metal detoxification, protection against herbivores and pathogens (Ortas and Rafique, 2017; Makarov, 2019; Smith and Read, 2008; Ahanger *et al*., 2014; Abdel-Salam *et al*., 2018). These fungi improve crop productivity and play a vital role in the organic farming system and maintain soil health and fertility (Subashini *et al*., 2007; Mahdi *et al*., 2010; Hijri, 2016). They can be developed for bio-fertilization of sustainable agricultural production systems, thereby decreasing the inputs for agrochemicals and protecting human and environmental health.

Bio-inoculants are individual strains or consortia of known microbes and play a significant role in plant growth when added to soil or seed coating (Owen *et al*. 2015). Being naturally occurring, bio-inoculants improve soil health and plant growth. Further, bio-inoculant formulations may be in the form of wettable powder (65%), granules (25%), and bacterial liquid suspensions (10%) that contains AM spores and mycorrhizal root fragments (Marin, 2005; Basiru *et al*., 2021). Bio-inoculant formulations based on microbial function can be used for many soil types and cropping systems (Roesti *et al*., 2006; Ahmad *et al*., 2013). Of late, research has been focused on AM fungi as bio-inoculant to produce high-quality inoculum (Ijdo *et al*., 2011). However, a sustainable agricultural system wherein chemical fertilizers are completely replaced requires continuous large-scale production of high-quality AM inoculum. Further, viability, germination, and colonization potential are necessary for a bio-inoculant.

Another essential characteristic of bio-inoculant production is the choice of organic carrier formulation. The carrier should hold the bio-inoculant in an excellent physiological condition. The resultant formulation should have good shelf life and good dissolution capability in the soil system. Besides, moisture absorption capacity, free lump-formation,

pH buffering capacity, and good adhesion to seed coating (Hegde and Brahmaprakash, 1992) are essential features that need to be considered.

For inoculum production, isolated spores, a mixture of spores, and mycorrhizal root pieces may be used as starter cultures (Gaur and Adholeya, 2000). In monoxenic culture, mycelia, spores, and highly colonized roots are obtained within a few months of inoculation (Declerck *et al*., 1998). In large-scale production and propagation of AM fungi, substrates are either used in pure or mixed forms. Some of the substrates employed so far include pure sand (Millner and Kitt, 1992), peat, glass beads (Lee and George, 2005; Neumann and George, 2005), vermiculite (Douds *et al*., 2006), perlite, compost, and calcinated clay. Further, the particle size of the substrate is essential for adequate drainage humidity and aeration. Sterilization of bio-inoculant carrier media is vital to prevent the growth of any possible contaminants (Wiseman *et al*., 2009).

Along with AM fungal bio-inoculants, rhizobial inoculants are also essential members of biofertilizers. They are mainly associated with legumes and vital in integrated soil fertility management. External application of rhizobial inoculants to the soil helps to enhance yield (Thuita *et al*., 2012). Further, improved rhizobial inoculants are inexpensive and non-polluting. They increase the efficiency of N delivery to the legume in the agro-ecosystem. If efficient and feasible carrier materials are used for such rhizobial inoculants, further enhancement in bio-fertilization can be achieved. It is believed that plant growth-promoting microorganisms (PGPMs) such as rhizobacteria-based inoculum are easy to prepare with an economical carrier material (Aseri *et al*., 2008; Prasad *et al*., 2012). Talc is a natural mineral known as steatite or soapstone, referred to as magnesium silicate $Mg_3Si_4O_{10}(OH)_2$. It has very low moisture equilibrium and relative hydrophobicity, low moisture absorption, and chemical inertness and prevents the formation of hydrate bridges that enable more extended storage periods (Reddy, 2012).

The talc-based formulation of *Bradyrhizobium* strain was suggested by Ramakrishnan *et al*., (1994). Some studies have shown that for bio-inoculant formulations, the two inorganic carrier materials, such as vermiculite and talcum powder, can be used due to their growth promotion and disease resistance characteristics (Bashan, 1998; Vidhyasekaran *et al*., 1997). In carrier bio-formulation, the use of gum arabic, carboxymethylcellulose (CMC), glycerol or polyethyleneglycol (PEG), sucrose solution,

and vegetable oil are commonly used as adhesive agents (Temprano *et al*., 2002). CMC is a cellulose-derived ester with a long chain of anhydroglucose, a highly hygroscopic, viscous polymer, and nontoxic to humans (Sanz *et al*., 2005). It gives stability to the formed gel (Suvorova *et al*., 1999).

Further, the bacterial carrier-based formulation in liquid, solid, and granules has been established for applications in the agricultural, industrial and pharmaceutical sectors. Due to its static nature and easy availability as raw material from soapstone industries, talc is used as a carrier for formulation development. The present study highlights the preparation of carrier formulation of effective inoculum with an increased number of propagules.

8.2: MATERIALS AND METHODS

8.2.1: Assessment of germination potential of monoxenically produced spores

The assessment of germination potential of monoxenically produced spores of *Gi. decipiens* was carried out for its utilization as carrier-based inoculum. The monoxenically produced spores of *Gi. decipiens* were inoculated on Modified Strullu-Romand (MSR) without sucrose.

8.2.1. I: Extraction of monoxenically produced AM propagules

The extraction of monoxenically produced AM fungal propagules from the MSR medium was carried out using the procedure of Cranenbrouck *et al*., (2005), described in Chapter 7. The extracted spores and attached extra-radical mycelium were then inoculated on a fresh MSR medium to study their germination potential.

8.2.2: Selection and preparation of efficient carrier formulation

For carrier formulation, a mixture of substrates consisting of vermiculite: cow dung powder: wood powder: wood ash in the proportion of 20:8:2:1 (64.51%: 25.80%: 6.45%: 3.22%) was employed (Rodrigues and Rodrigues, 2017). Vermiculite was obtained from Sri Ramamaruthi Vermiculite Mines, Chennai, India, and the average size of vermiculite particles was 40-70 µm. The plant source of wood powder and wood ash was *Mangifera indica* L. (Anacardiaceae). The wood powder was washed 3-4 times with tap water, followed by oven drying and autoclaving. The carrier material was sterilized by autoclaving for two consecutive days at 121^0C for two hours. For Physico-chemical

characterization of the carrier material, samples were analyzed at the soil analysis laboratory, ICAR-Central Coastal Agricultural Research Institute Ela, Old Goa, as described in Chapter 5. Also, Boron (B) was quantified by using the hot water-soluble method (Berger and Truog, 1939).

8.2.2. I: Carrier-based mass multiplication

The mass multiplication experiment was set up using deep cell plug trays for three months. Twenty-five *in vitro* produced *Gi. decipiens* spores and colonized transformed chicory and *Linum* roots were used as inocula for each deep cell plug containing the carrier formulations. These were then planted (under sterile conditions) with pre-rooted cuttings of *P. scutellarioides.* The experiment was conducted in triplicate. The plants were maintained in the phytotron (Daihan Labtech, LGC-6201G) at 260 lux (16 h photoperiod), 26^0C, 41.1 % humidity, and 100 ppm CO_2. Hoagland's solution (Hoagland and Amon, 1950) minus P was applied every 20 days to the plants. Replicate of each plug trays were kept in a single polypropylene laboratory tray. Further, after 90 days of the experiment, the inoculum of AM fungal species was stored in *Ziploc* polyethylene bags at room temperature (28 ± 2^0C). To maximise the formulation's shelf life, care was taken to ensure the absence of free moisture inside the bags.

8.2.2. II: Processing of root segments for AM fungal colonization

Assessment of AM colonization in roots of *Plectranthus scutellarioides* (L.) R.Br. was carried out using Trypan blue staining technique after 45 days of growth (Phillips and Hayman, 1970), as described in Chapter 5. All microscopic observations were made using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, l00x magnification) and Olympus stereo microscope SZ2-ILST (45x magnification). Micrographs were imaged using Olympus DP 12- 2 and Nikon Digital Sight DS-U3 digital cameras and were not digitally edited.

8.2.3: Preparation of suitable carrier and bacterial suspension

Talcum powder was obtained from Starke and Co. Pvt. Ltd., India. The average size of the talcum powder particles was 50-80µm. Bacterial cells were harvested and centrifuged at 6000 rpm for 15 minutes and re-suspended in phosphate buffer (0.01M, pH 7.0). These isolates were kept at -80^0C in 44% glycerol, and cells from stocks were first grown on YEMA. The inoculum was produced by transferring one loopful of purified bacterial

culture to 100 mL of YEM broth taken in a 250 mL Erlenmeyer flask and incubating at room temperature (28±2^{0}C) on a shaker at 150 rpm for 48 h.

8.2.3.I: Preparation of talc-based formulation of *Bradyrhizobium* strain

The carrier material was steam-sterilized at 121^{0}C for at least 30 minutes on two subsequent days and dried aseptically in glass trays for 12 hours at 50^{0}C before use (Ardakani *et al*., 2010). After 48 hours of incubation, the broth culture of *Bradyrhizobium* was used to prepare talc-based formulations. In a metal tray, 100 g of purified talc powder was taken, and to that, 40 mL of bacterial suspension was added along with 1.5 g calcium carbonate (adjusted to neutral pH) and 1 g carboxymethylcellulose (CMC adhesive) under sterile conditions, following the method described by Vidhyasekaran and Muthuamilan, (1995). The final formulation contained 10^6 CFU mL^{-1} of *Bradyrhizobium* strain. The formulation was shade-dried to reduce the moisture content to less than 20% and then packed in polyethylene bags, sealed, and stored at room temperature (25±2^{0}C).

8.2.3. II: Determination of shelf-life/viability

The serial dilution method ascertained the viability of bacterial isolates in the formulation. Talc-based formulation (50 mg) was dissolved in 1 mL of sterile distilled water in an Eppendorf tube. Later, 10 µl of the suspension was diluted using 990 µl of sterilized distilled water. The diluted sample was plated on a YEMA medium. The plates were incubated at (28±2^{0}C), and viability was checked after 2 and 4 days.

8.3: RESULTS AND DISCUSSION

8.3.1: Germination and colonization potential of monoxenically produced spores

It was observed that *Gi. decipiens* spores germinated within three days time intervals in monoxenic conditions. The study further revealed that *in vitro* produced spores of *Gi. decipiens* retained maximum germination potential for up to 24 weeks **(Plate 8.1)**. The spore, along with its extra-radical hyphae, could colonize roots. Intra-radical structure formation in cortical root cells initiated once the spore germinated and root penetration occurred as observed earlier by Sbrana and Giovanetti, (2005). The presence of hyphae, arbuscules, and extra-radical spores indicated the successful colonization of *P. scutellarioides* roots by *Gi. decipiens*, as reported in an earlier study by Hale *et al*., (2015).

Further, AM fungal monoxenic culture production method proposed by Strullu and Romand, (1986) was followed, wherein successful isolation of colonized roots and re-association with other root systems was carried out. The monoxenic cultures produced were maintained contamination-free through sub-cultivation as suggested by Plenchette *et al*., (1996), Strullu *et al*., (1997), Declerck *et al*., (1998), and Ijdo *et al*., (2011). Further, the co-culture of AM fungal consortium rather than a single organism was done to make it more beneficial to plants, as suggested by Daft (1983).

8.3.2: Physico-chemical characterization of materials used for carrier formulation

The Physico-chemical nutrient analysis of various carriers and their formulation is presented in **Table 8.1**. The pH value of the various carrier components ranged between 5.42-11.19, with the carrier formulation having a pH of 8.22. *Gi. decipiens* with a wide pH tolerance were successfully able to grow at this pH. The nutrient parameters varied in different carrier materials, with the highest macro- and micro-nutrient (except Cu) content in cow dung. Further, wood ash contained insignificant amounts of N but high amounts of Cu.

Accordingly to Dodd and Jeffries (1986), arbuscule formation significantly increases when nutrient demand by the host plant increases. High nutrient content in the carrier can increase AM fungal root colonization and sporulation (Rodrigues and Rodrigues, 2017). Therefore, the present study supports carrier formulation usage with the benefits of macro- and micro-nutrients, improved water retention capacity, increased substrate permeability, and the potential to maintain AM fungal propagule inoculum.

8.3.3: Carrier formulation using *in vitro* produced colonized inoculum

In the present study promising organic carrier substrates *viz*., vermiculite, cow dung powder, wood powder, and wood ash were used for the formulations. It is also recommended for AM fungal bio-inoculants formulation at commercial production. Commercially, peat is widely used as a carrier material (Yardin *et al*., 2000). However, the availability of peat is limited as a natural resource. Several researchers have proposed soil-based culture and carrier-based inocula for AM fungal inocula production (Adholeya, 2003). Millner and Kitt (1992) have recommended carrier inoculum containing a mixture of air-dried substrate *viz.,* sand, vermiculite, and soil-rite. Adholeya *et al*., (2005) reported AM fungal inoculum in powdered tablets, pellets or granules, gel beds, and ball form.

In the present study, mass multiplied *in vitro* produced spores of *Gi. decipiens* and attached extra-radical mycelia were used separately as inoculum and cultured under controlled laboratory conditions **(Plate 8.2)**. Further, the Wet sieving and decanting technique (Gerdemann and Nicolson, 1963) was employed to estimate the spore density, as described in Chapter 5. A spore density of 130 spores $100g^{-1}$ of carrier inoculum was recorded.

Although the greenhouse or pot culture technique is currently used to produce AM fungal inocula (Douds *et al*., 2000; Gianinazzi and Vosatka, 2004; Singh, 2002), the *in vitro* culture technique is considered the most effective monoxenic culture method. In this technique, Ri T-DNA transformed host mycorrhizal roots grown in split plate method (Becard and Piche, 1992; St-Arnand *et al*., 1996), in a limited space produces high concentration of propagules (Dalpe and Monreal, 2004; Adholeya *et al*., 2005). However, the amount of AM fungal propagules produced depends on the type of Ri T-DNA transformed host mycorrhizal roots employed (Tiwari and Adholeya, 2003).

In the present study, the carrier formulation ratio 20:8:2:1 (vermiculite: cow dung powder: wood powder: wood ash) reported 100% colonization and significant interaction with the host plant. The present results conform with the findings of Rodrigues and Rodrigues (2017). Further, the research assessed that vermiculite-based media shows a higher number of AM fungal propagules than perlite or horticultural potting media where inoculum is mixed with compost (Douds *et al*., 2010). For several decades vermiculite has been well studied and used as carrier material (Sangeetha and Stella, 2012). However, organic amendments rich in nutrients were observed to increase colonization and sporulation, leading to significant inoculum production (Silva *et al*., 2005; Perner *et al*., 2006; Coelho, 2014).

Table 8.1: Physico-chemical parameters of carrier and carrier composition.

Carrier material	pH	EC (dS/m)	OC %	Macro-nutrients (kg/ha)			Micro-nutrients (mg/kg)				
				N	P	K	Zn	Fe	Mn	Cu	B
Vermiculite	6.08±0.6	0.17±0.1	0.82±0.4	125.23±0.82	80.51±0.03	178.41±0.24	0.65±0.01	1.39±0.21	16.26±0.02	0.25±0.02	1.28±0.05
Cow dung powder	6.01±0.7	2.80± 1.0	4.52±1.1	689.92±0.80	449.41±0.28	462.55±0.19	6.82±0.03	12.46±0.13	24.35±0.1	1.30±0.01	48.51±0.01
Wood powder	5.42±1.0	0.90±0.5	2.24±0.7	227.42±0.44	107.70±0.19	168.83±0.05	3.26±0.09	2.89±0.05	1.82±0.1	0.25±0.03	12.56±0.1
Wood ash	11.19±1.1	12.24±1.4	2.21±0.4	118.35±0.29	214.88±0.05	698.43±0.04	4.28±0.08	12.55±0.47	8.35±0.2	23.30±0.12	24. 32±0.03
Carrier Composition	8.22±0.02	1.74±0.05	2.64±0.01	382.24±0.12	446.34±0.19	238.32±0.04	3.85±0.02	7.47±0.01	20.65±0.01	1.77±0.01	6.24±0.5

Legend: All values are mean of three readings. **EC**= Electrical conductivity, **OC**= Organic carbon, **N**= Nitrogen, **P**= Phosphorus, **K**= Potassium, **Zn**= Zinc, **Fe**= Iron, **Mn**= Manganese, Copper= **Cu**, **B**= Boron

AM fungal inocula can be better stored in carrier formulations. Kuszala and Gianinazzi-Pearson (2011) reported that propagules of AM genera, namely *Acaulospora*, *Glomus*, *Scutellospora*, and *Gigaspora*, isolated from 6-11 months old pot culture could be either stored at 4^0C in osmosed water or 27^0C or 37^0C in carrier formulations. Further, at the International Culture Collection of Vesicular Arbuscular Mycorrhizal (INVAM), depending on AM fungal genera, propagules from pot cultures are stored in the dried substrate or soil at 4^0C. At the same time, carrier-based AM fungal inocula are maintained at 25^0C. Rodrigues and Rodrigues (2018) also observed that carrier-based formulation is a better inoculum storage method than other available methods. Similar results with vermiculite formulations were obtained by de Santana *et al*., (2014) and Berruti *et al*., (2014). Carrier formulations sustain good shelf-life, can be stored at room temperature, retain moisture content, and are effective pre- or post-application (Hong *et al*., 2005; Friesen *et al*., 2006). The *in vitro* produced inocula stored at 25^0C remained viable for six months. However, further experiments may be needed to test the suitability of the carrier formulation developed for long-term preservation and reproducibility in other AM fungal species.

8.3.4: Talc-based formulation using *Bradyrhizobium* strain

The present study revealed that *Bradyrhizobium* species is responsible for cowpea nodulation. But various studies have reported both *Bradyrhizobium* and *Rhizobium* ability to form nodulation in cowpea (Mpepereki *et al*., 1996; Gronemeyer *et al*., 2014). Nodules forming bacteria take 2-4 days to grow on YEMA plates and are considered fast-growing. At the same time, fast-growing (*Rhizobium*) and slow-growing (*Bradyrhizobium*) species have been reported by Chidebe *et al*., (2018). For the preparation of consortium, a concentration of 10^6 CFU mL^{-1} in broth culture was sufficient to colonize *V. unguiculata* (L) Walp. during the application period **(Plate 8.3)**. In some rhizobacteria, *Pseudomonas fluorescens* (strain Pf1 and Pf2) maintained the highest population of 1.8×10^9 CFU g^{-1} at six-month period in talc-based carrier formulation (Gade *et al*., 2014).

Studies have revealed the importance of carrier-based formulation in maintaining the viability of inocula in a physiologically competent state and providing a definite shelf life before applying to the soil (Bashan, 1998). In general, for plant growth-promoting microbes, several substrates have been used for carrier formulation, *viz.*, peat, talc,

charcoal, farmyard manure, cellulose powder, vermicompost, lignite, compost, bagasse, and press mud (Gopalakrishnan *et al*., 2016).

The present study focuses on preparing bacterial inoculum in powdered form, as talc powder is known to have the potential as a carrier for rhizobacteria formulation (Kloepper and Schroth, 1981). Talc is widely used as a bacterial carrier inoculant, and its application to soil was reported to be effective against plant diseases (Meena *et al*., 2002; EI-Hassan and Gowen, 2006). Studies have reported that carrier-based formulations of sawdust, rice husk and talc powder are significant for certain rhizobacteria and increase the shelf life by up to 9 months (Chakraborty *et al*., 2013). About talc-based formulation of bacterial inocula, an alternative study suggested that, with the addition of N-fixing and P-solubilizing bacteria, vermicompost alone as a carrier increased the amount of N and P availability (Kumar and Singh, 2001). Another alternative carrier for rhizobial bacteria is a biochar amendment that improves soil fertility (Egamberdieva *et al*., 2017).

In the present study, talc-based inoculant preparation was carried out and stored at an ambient temperature ranging from 25-30^{0}C. In carrier formulation, the maturation of bacterium reached to required standards. The carrier formulation was found to have a significant shelf-life period of six months. Previous studies have shown that talc-based formulation stored at 26^{0}C had the most comprehensive stability studies (Sadi and Mousad, 2012; Sangeetha and Stella, 2012). It was also observed from previous studies that talc-based carrier formulation of *Pseudomonas* strain showed greater viability at room temperature for several months and benefits colonization potential at rhizosphere (Novinscak and Filion, 2020).

In the present study, carboxymethylcellulose (CMC) is used as an additive substrate for the talc-based carrier formulation and showed a high ability to sustain concentration up to six months of storage. Furthermore, CMC and starch blend allows for improved gel feature, higher hygroscopicity, and viscosity (Rohr, 2007). It is a cheap inoculant carrier material used in the food and pharmaceutical industries. Starch has been used as a carrier or additive in formulation with dried beds or liquid core capsules (Kim *et al*., 2005). Krishnamurthy and Gnanamanickam (1998) developed a talc-based formulation of *Pseudomonas fluorescens* to manage rice blast caused by *Pyricularia grisea*, in which methylcellulose and talc were mixed at a 1:4 ratio. They blended the mixture with an equal

volume of a bacterial suspension at a 1010 CFU mL^{-1} concentration. Several studies observed that in carrier bio-formulation, talc and bentonite are effectively significant compared to peat and rice bran (Jorjani *et al*., 2011).

In the present study, 1 g of CMC:100 g talc powder and 40 mL liquid broth of *Bradyrhizobium* strain were used for the carrier formulation to give significant sustainable inoculum for further experimental study. Such a bio-formulation has a shelf life of up to six months at room temperature or in laboratory conditions. Further, mass production at a commercial scale will significantly add value to this bio-consortium.

8.4: CONCLUSION

The monoxenic culture of *Gi. decipiens* was successfully established in transformed roots of *L. usitatissimum* Further, the culture produced BAS and spores, highly desirable characters for considering an AMF strain for biofertilizer inoculum production. Effectively mass multiplication was carried out under laboratory conditions using *P. scutellarioides* as the host plant. Bio-formulation was prepared using a carrier component in the ratio 20:8:2:1. The bio-formulation prepared was successfully packed and stored at room temperature. The culture is being further developed as an inoculum for its application as a biofertilizer.

In the present study, N fixing bacteria were isolated from the root nodules of *V. unguiculata* using the YEMA medium. The isolated bacteria are a strain of *Bradyrhizobium*. It grows within 2-4 days time intervals in culture media. As a potential nitrogen-fixing bacteria, talc (CMC as additive) based formulation was prepared and maintained. The application of this bacterial formulation may benefit cowpea farmers in agricultural fields.

CHAPTER 9: Evaluation of promising AM fungi and Rhizobial strains for increased yield in cowpea

9.1: INTRODUCTION

AM fungi are widespread in natural forests and agricultural ecosystems. They found that AM application improves soil properties and enhances root colonization, increasing plant growth and productivity (Curaqueo *et al*., 2011; Bedini *et al*., 2013). Studies have also indicated that native species or isolates are more effective than exotic species (Tchabi *et al*., 2010).

In legume crops, rhizobia and AM fungal inoculants can be used as alternative sources of N and P, respectively. Several researchers have highlighted the significance of AM fungi as bio-inoculants in the agricultural system (Ijdo *et al*., 2011; de Souza *et al*., 2010). However, the symbiosis of rhizobia and AM fungi in legumes significantly improves mineral nutrition in plants (Ossler *et al*., 2015). Further, legume nodulation and N fixation directly relate to P supply (Pereira and Bliss, 1989; Jakobsen, 1985).

Sometimes the screening and evaluation of leguminous plants in the field condition are necessary during the crop growing period to overcome the low efficiency of native rhizobial strains. Further, crop productivity can be increased by using efficient N-fixing inoculant strains. In a sustainable system, renewable resources mainly focus on BNF supplying N for agriculture (Dixon and Wheeler, 1986; Peoples *et al*., 1995). Further, legume-rhizobia N fixation provides significant N inputs for agriculture (Lanier *et al*., 2005). Besides, the application of biofertilizers helps overcome water-related stress, thereby helping in maintaining a sustainable agroecosystem (Oliveira *et al*., 2017; Sharma *et al*., 2018).

The promising microbial inoculants such as AM fungi and rhizobia improve the quality of leguminous crops, have a long-lasting effect in the soil, increase rhizobial infection rate, and benefit the ecosystem (Omomowo *et al*., 2009). Further, organic matter and organophosphate-bound ions from the soil are made available to the plant using microbial inoculants. Research has also shown that the negative impact of chemical fertilizer can be overcome by using single and co-inoculation of rhizobia and AM fungi (Abd-Alla *et al*., 2014). Researchers have reported that single and co-inoculation of rhizobia and AM fungi

in cowpea and chickpea increases the photosynthetic capacity of chlorophyll a and chlorophyll b (Oliveira *et al*., 2005; Bejandi *et al*., 2012). It has also been reported that legume crops with rhizobia and AM fungi can overcome various environmental stresses. Also, the cost of N and P fertilizers can be reduced by dual inoculation (Bonfante and Anca, 2009).

It has been reported that the increase in AM fungal colonization, root surface area, and plant hyphal penetration is due to PGPR phytohormones secretion (Kumar *et al*., 2015). PGPR has specific phosphate solubilization, soil aggregate stabilization, and growth promotion (Vafadar *et al*., 2014; Guinazu *et al*., 2010; Arora *et al*., 2010). An earlier study has reported that the presence of PGPRs highly influences AM fungal symbiotic association in plants (McNear, 2013). Nutrient retention in the plant tissue and mobility to facilitate soil nutrient concentration ratios depend on AM fungi and PGPR combination (Singh *et al*., 2011).

In the agro-ecosystem of the Goa region, extensive cultivation of cowpea has demonstrated the importance of the legume crop under rice fallow conditions. Cowpea crop has a high demand amongst the locals in Goa, and the current traditional cultivation practices do not comply with the supply needs. Generally, poor yield and increased susceptibility to major diseases and pests decrease the revenue generation opportunities of the farmers. Therefore, this chapter aims to evaluate the effectiveness of *in vitro* produced carrier-based bio-inocula of AM fungi, dual inoculation with *Bradyrhizobium* sp., and PGPR on the growth and yield of cowpea under field conditions.

9.2: MATERIALS AND METHODS

9.2.1: Field experimental design

The field experiment was carried out during 2018-2019 in sandy, loamy soils at Colva village in South Goa, situated at Latitude $15^0$17'12.88"N and Longitude $73^0$54'43.92"E. The selected field was ploughed and leveled using a tractor. The field experiments consisted of randomized block design comprising three replicates in rows (R_1, R_2, and R_3), with plots of 6 × 5 m with a 60 × 60 cm spacing. The variety used in this trial was Goa cowpea-3, the most preferred variety. Healthy cowpea seeds were surface washed and soaked overnight in sterile distilled water to soften the seed coats for sowing purposes.

Details of treatments are depicted in **Table 9.)**. Talc-based inoculum of *Bradyrhizobium* strain (GUBRS2) and *Bacillus methylotrophicus* (Goa Bio-1 culture) were used for seed coating @30 g kg^{-1} of seeds with a gap of 15 minutes for both the bio-inoculants. The seeds were sown at a depth of 5 cm. For AM treatments (T_2, T_5, T_6, and T_8), 100 g of *in vitro* produced inoculum with carrier formulation consisting of vermiculite, cow dung powder, wood powder, and wood ash in the proportion of 20:8:2:1 (Rodrigues and Rodrigues, 2017) was added. A total of 240 seeds were sown in the entire experimental plot. The plots were irrigated after sowing, at flowering and pod development stages. Timely weed management practices were followed to raise a good crop.

Table 9.1: Treatments using selected consortium of bio-inoculants.

Code	Treatments
T_1	Control
T_2	*Gigaspora decipiens*
T_3	*Bradyrhizobium* sp.
T_4	*Bacillus methylotrophicus* (Goa Bio-1 culture)
T_5	*Gigaspora decipiens* + *Bradyrhizobium* sp.
T_6	*Gigaspora decipiens* + *B. methylotrophicus*
T_7	*Bradyrhizobium* sp. + *Bacillus methylotrophicus*
T_8	*Gigaspora decipiens* + *Bradyrhizobium* sp. + *Bacillus methylotrophicus*

Table 9.2: Randomized plot design R_1

Treatments	Seeds Number									
R_1 T_1	*	*	*	*	*	*	*	*	*	*
R_1 T_2	*	*	*	*	*	*	*	*	*	*
R_1 T_3	*	*	*	*	*	*	*	*	*	*
R_1 T_4	*	*	*	*	*	*	*	*	*	*
R_1 T_5	*	*	*	*	*	*	*	*	*	*
R_1 T_6	*	*	*	*	*	*	*	*	*	*
R_1 T_7	*	*	*	*	*	*	*	*	*	*
R_1 T_8	*	*	*	*	*	*	*	*	*	*

Legend: *= number of seeds sown per treatment

Table 9.3: Randomized plot design R_2

Treatments	Seeds Number									
R_2 T_6	*	*	*	*	*	*	*	*	*	*
R_2 T_5	*	*	*	*	*	*	*	*	*	*
R_2 T_1	*	*	*	*	*	*	*	*	*	*
R_2 T_2	*	*	*	*	*	*	*	*	*	*
R_2 T_4	*	*	*	*	*	*	*	*	*	*
R_2 T_8	*	*	*	*	*	*	*	*	*	*
R_2 T_7	*	*	*	*	*	*	*	*	*	*
R_2 T_3	*	*	*	*	*	*	*	*	*	*

Legend: *= number of seeds sown per treatment

Table 9.4: Randomized plot design R_3

Treatments	Seeds Number									
R_3 T_4	*	*	*	*	*	*	*	*	*	*
R_3 T_8	*	*	*	*	*	*	*	*	*	*
R_3 T_2	*	*	*	*	*	*	*	*	*	*
R_3 T_6	*	*	*	*	*	*	*	*	*	*
R_3 T_5	*	*	*	*	*	*	*	*	*	*
R_3 T_1	*	*	*	*	*	*	*	*	*	*
R_3 T_7	*	*	*	*	*	*	*	*	*	*
R_3 T_3	*	*	*	*	*	*	*	*	*	*

Legend: *= number of seeds sown per treatment

9.2.2: Plant growth and harvest stage parameters measurement

In field conditions, several parameters were evaluated at various stages of plant growth. The parameters include plant height, number of branches per plant, number of days required for flowering, number of leaves per plant, number of pods per plant, pod length, number of seeds per pod, number of seeds per plant, seed weight per plant and straw yield at harvesting stage.

9.2.3: Processing root segments for AM fungal colonization

The root system of *V. unguiculata* from three randomly selected plants (per inoculated treatments T_2, T_5, T_6, and T_8) was assessed for AM fungal colonization 45 days after sowing (DAS) by using Trypan blue staining technique (Phillips and Hayman, 1970) as described in Chapter 5.

9.2.4: Statistical analysis

Differences in the replicates and treatments were assessed using two-way ANOVA with significant differences at p values <0.05. Pearson's correlation analysis was performed to test relationships between the analyzed parameters. Correlation coefficients (r) were considered significant at p values <0.05. These analyses were performed using STATISTICA software (version 8.0, StatSoft, USA). All microscope observations were made using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (400x, l000x magnification) and Olympus stereo microscope SZ2-ILST (45x magnification). Micrographs were imaged by Nikon Digital Sight DS-U3 digital camera and were not digitally edited.

9.3: RESULTS AND DISCUSSION

9.3.1: Root colonization

In the present study, the bioinoculant treatments depicted in **Table 9.1** were used for treating the pre-sowed seed material in the randomized block design (RBD) (**Plate 9.1; Tables 9.2, 9.3, 9.4)**. For successful agriculture, it is essential to use microorganisms that improve the nutrient availability of the plant (Figueiredo *et al*., 2008). In the experimental cowpea field, plants showed dense root colonization 45 days after sowing the seeds. Compared to un-inoculated control, hyphae and arbuscules were observed in all the inoculated treatments (T2, T5, T6, and T8). The presence of symbiotic N-fixing bacteria influences AM fungal colonization and community structure in plant roots (Omirou *et al*., 2016). An increase in the availability of P and N due to the symbiotic interaction of AM fungi and *Bradyrhizobium japanicum* strain resulted in improved plant growth (Abd-Alla *et al*., 2014). The interaction of AM fungi and rhizobia is highly beneficial to the plant under drought conditions (Ruiz-Lozano *et al.*, 2001).

The results of the root colonization in *V. unguiculata* are depicted in **Plate 9.2**. The extent of root colonization varied significantly among the different AM treatments. In the present study, the AM fungal colonization was maximum during the flowering and fruiting stage, and subsequently, sporulation occurred at the end of the plant life cycle. In a similar study, Bever *et al*., (1996) concluded that AM fungal developmental stages in the cowpea root system influence the structure and composition of AM fungal communities. Mummey *et*

al., (2009) suggested that sometimes indigenous AM fungal species in the rhizosphere may compete with the applied AM fungus.

9.3.2: Plant vegetative growth and harvest parameters response

In the present study, the biofertilizers in the form of AM fungi, nitrogen-fixing bacteria, and plant growth-promoting bacteria (PGPB) were used to study the improvement in seed yield (**Plate 9.3**). The study aimed to maximize yield potential by providing a high number of viable rhizobia to the rhizosphere to allow rapid colonization, nodulation, and atmospheric N fixation by a selected inoculant strain. Earlier reports highlighted that co-inoculation of rhizobial strains with AM fungi or rhizobia strains with plant growth-promoting bacteria (PGPB) improved crop yield (Lima *et al*., 2011; Omirou *et al*., 2016; Araujo *et al*., 2010; Rodrigues *et al*., 2013). The inoculation results in increased output and is also an environmentally friendly technology that helps overcome the harmful effects of chemical fertilizers (Abd-Alla *et al*., 2014).

According to Antunes (2005), the interaction between AM fungi and *Bradyrhizobium* is due to the secretion of flavonoids by the host plant, which stimulates hyphal growth in AM fungi and nodule formation in *Bradyrhizobium*. The mutualistic symbiosis between AM fungi, plant growth-promoting bacteria (PGPB), and legume rhizobial association results in a significant increase in crop yield (Bonfante and Anca, 2009).

Cowpea is cultivated as a rotational crop in fallow rice fields in Goa. According to Datta *et al.*, (1982), the application of biofertilizers during seed treatment is an essential aspect of improving the yield by 5 to 30 percent. The present study revealed that inoculation with bio-inoculants significantly affected the growth and yield characteristics compared to control (**Tables 9.5, 9.6; Plates 9.4, 9.5**). Studies have also revealed that rhizobial inoculation improves seed yield by increasing the pod number, seeds per pod, and seed weight (Denton *et al*., 2013; Youseif *et al*., 2017). Studies have revealed that a successful increase in yield under low fertility and marginal soil conditions can be achieved by adopting crop rotation (Bell *et al*., 2017).

The statistical analysis of the total number of days required for flowering revealed not much difference between the inoculated treatments compared to the control. The results show that single inoculation with AM fungi, *Bradyrhizobium* sp., and PGPB also

significantly increased the vegetative characteristics of cowpea plants. Souchie *et al*., (2007) reported that Plant Growth-Promoting Bacteria (PGPB) suppresses plant diseases, increases phytohormone concentration, solubilizes inorganic phosphate, and produces siderophores, oxidizes sulfur, and increases root permeability. Biological agents such as PGPR can effectively produce phytohormones and siderophores and efficiently use microbial inoculants in seed treatment (Berg, 2009; Verbon and Liberman, 2016). Earlier studies also confirm that plant qualities such as nutrient uptake, N fixation, plant growth, yield, and crop quality are enhanced when inoculated with PGPR (Yadav and Verma, 2014; Günes *et al*., 2014; Stefan *et al*., 2013). It is also reported that inoculation with *Bradyrhizobia* in low-N soils of Ethiopia improves plant growth, nodulation, and grain yield in cowpea (Yoseph *et al*., 2017).

Studies have also shown that cowpea seed treatment with *Bacillus* strain improves seed germination and other yield parameters and benefits P solubilization, ACC deaminase, and antifungal activity (Nain *et al*., 2012). Compared to *Pseudomonas* species the *Bacillus* species produce heat-resistant endospores, considered efficient and stable biological products that can overcome the seed treatment stress (Yánez -Mendizabal *et al*., 2012). The present study revealed that the growth of cowpea plants is significantly higher in inoculated treatments than in control. However, a significant increase was recorded in branches and leaf number per plant between single and co-inoculation treatment than the control plants (**Fig. 9.1: 1a-1d**). Plant receiving single and co-inoculation with *Bradyrhizobium* species, PGPR, or AM species positively affected all productivity parameters analyzed. Similarly, the number of pods per plant inoculated with *Bradyrhizobium*, PGPR, and AM species was significantly higher than control plants. The study revealed a significant difference in pod length and number of seeds per pod in the various treatments compared to the control.

The present study revealed that inoculation with *Bradyrhizobium* species, PGPR, and AM species recorded an increased seed number than the control. Batista *et al*., (2017) also reported a significant increase in pods per plant and grain yield with single and combined inoculation with *Rhizobium tropici* and *R. leguminosarum*. The seed weight per plant was positively affected by single and combined inoculations and significantly increased compared to control treatments. Likewise, the straw yield was higher in inoculated treatments than in control plants (**Fig. 9.1: 2a-2f**). ANOVA shows significant variation in

plant height among the treatments (ANOVA P= 0.0321, F= 3.15) with maximum height in T_8 compared to T_1 treatment (**Table 9.7**). The maximum number of pods per plant was recorded in the T_8 treatment compared to control. An increase in the number of pods per plant significantly increased the yield.

The Spearman's rank correlation results are presented in (**Table 9.8**). The study indicated a strong positive correlation between plant height and number of primary branches (r^2= 0.62, n= 8, p<0.05) and leaf number (r^2= 0.63, n= 8, p<0.05). Earlier reports on cowpea have also shown that the leaf number per plant, pod number, and yield are positively correlated (Akundabweni, 1990). Previous bioinoculation studies have shown that both in sterilized and non-sterilized soil conditions, single and dual inoculation with the *Bradyrhizobium* and NFB significantly increased cowpea shoot biomass (Omirou *et al*., 2016).

Overall, a significant positive correlation between plant height and pod number (r^2= 0.77, n= 8, p<0.05), seeds number (r^2= 0.76, n= 8, p<0.05), seed yield (r^2= 0.76, n= 8, p<0.05) and straw yield (r^2= 0.55, n= 8, p<0.05) was observed. In Nigeria, a cowpea field trial involving inoculation with *Glomus versiforme* and *Trichoderma hazianum* reported significant increase in plant height, fresh and dry root and shoot weight, resistance to powdery mildew disease, and improved yield (Omomowo *et al*., 2018).

Oliveira *et al*., (2017) reported that the seed number and the total weight of seeds per plant in cowpea increased upon single inoculation with *Bradyrhizobium elkanii* and dual inoculation with *Rhizophagus irregularis* treatment. Number of pods per plant is positively correlated to seeds per plant (r^2= 0.97, n= 8, p< 0.05) and seed weight (r^2= 0.86, n= 8, p< 0.05). Peksen (2004) reported a positive correlation between the pod length and individual pod weight. In the present study, the time taken for flowering ranged from 51-54 days. The days taken for flowering were negatively correlated to most of the experimental parameters in the study.

Table 9.5: Effect of monoxenically produced carrier-based bioinocula of *Gi. decipiens*, talc bio-formulation of *Bradyrhizobium* sp. and *Bacillus methylotrophicus* on vegetative growth characteristics of *V. unguiculata* at 90 DAS (days after sowing).

Treatments	PHt (cm)	L/P	PrB
T_1	40.83±17.57	34.31±15.60	3.46±1.01
T_2	46.07±22.53	39.52±19.10	3.62±0.92
T_3	49.62±26.18	37.31±18.05	3.50±0.83
T_4	54.94±28.09	47.33±22.48	4.18±0.77
T_5	50.83±23.46	45.04±21.37	4.00±0.89
T_6	48.83±22.80	43.01±20.63	4.04±0.78
T_7	55.50±27.64	40.61±18.76	3.74±0.64
T_8	63.42±32.81	46.98±21.74	4.31±0.52

Legend: All values are means of 3 replicates; **PHt** (cm)= Plant Height, **L/P**= No. of leaves or leaflets/plant, **PrB**= Primary branches per plant, T_1: Control, T_2: *Gigaspora decipiens*, T_3: *Bradyrhizobium* sp., T_4: *Bacillus methylotrophicus* (Goa Bio-1 culture), T_5: *Gi. decipiens* + *Bradyrhizobium* sp., T_6: *Gi.decipiens* + *B. methylotrophicus*, T_7: *Bradyrhizobium* sp. + *B. methylotrophicus*, T_8: *Gi. decipiens* + *Bradyrhizobium* sp. + *B. methylotrophicus*.

Kandel *et al.*, (2019) opined that the variation in the time taken for flowering may be due to differences in agroclimatic conditions. The present study suggests the potential of improving cowpea seed yield by using bio-inoculants. In soybean production, plants inoculated with *Bradyrhizobium* and AM fungi significantly affected the N fixing capacity, solubilization, and mobilization of P uptake, enhancing plant growth, nutrition, and yield (Gitonga *et al*., 2021).

Table 9.6: Effect of monoxenically produced carrier-based bio-inoculum of *Gi. decipiens,* talc bio-formulation of *Bradyrhizobium* sp. and *Bacillus methylotrophicus* on yield characteristics in *V. unguiculata.*

Treatments	DFF	PL	P/P	S/P	S/PLt	SW	SY
T_1	54	18.93±2.62	8.00±1.57	12.00±1.35	93±21.71	680.84±5.33	89.97
T_2	53	19.38±1.69	11.00±1.82	12.00±0.75	138±27.38	997.76±6.85	112.07
T_3	53	19.90±1.65	11.00±2.59	12.00±1.13	135±35.34	983.05±8.73	100.27
T_4	53	19.41±1.74	13.00±1.81	12.00±1.16	157±26.05	1121.62±7.13	107.03
T_5	53	19.61±2.34	13.00±3.37	12.00±1.04	153±36.09	1111.30±8.34	110.13
T_6	53	19.62±1.65	11.00±3.51	13.00±1.41	137±44.52	989.18±10.25	111.60
T_7	53	19.62±1.65	12.00±1.56	11.00±1.11	138±21.97	1013.17±5.67	114.27
T_8	51	19.55±1.27	17.00±3.13	12.00±0.81	202±35.70	1462.32±8.58	126.23

Legend: All values are the mean of 3 replicates; **DFF**= Days taken for flowering, **PL** (cm)= Pod length, **P/P**= Number of pods/plant, **S/P**= Number of seeds/pod, **S/PLt**= Number of seeds/plant, **SW**= Seed weight in grams, **SY**= Straw yield in grams; T_1: Control, T_2: *Gigaspora decipiens*, T_3: *Bradyrhizobium* sp., T_4: *Bacillus methylotrophicus* (Goa Bio-1 culture), T_5: *Gi. decipiens* + *Bradyrhizobium* sp., T_6: *Gi. decipiens* + *B. methylotrophicus*, T_7: *Bradyrhizobium* sp. + *B.methylotrophicus*, T_8: *Gi. decipiens* + *Bradyrhizobium* sp. + *B. methylotrophicus*.

Table 9.7: Effect of monoxenically produced carrier-based bio-inoculum of *Gi. decipiens,* talc bio-formulation of *Bradyrhizobium* sp. and *Bacillus methylotrophicus* on growth and yield characteristic in *V. unguiculata* differentiated using ANOVA table.

Factors	Parameters	df	MS	F	p value
Replicates	DFF	2	3.04	2.14	0.1548
	PHt (cm)	2	57.80	1.31	0.3001
	PrB	2	0.04	0.35	0.7128
	L/P	2	17.72	1.18	0.3375
	P/P	2	5.52	1.14	0.3471
	PL (cm)	2	0.51	1.10	0.3586
	S/P	2	1.59	3.22	0.0707
	S/PLt	2	802.58	1.18	0.3350
	SW (g)	2	118.03	1.33	0.2950
	SY (g)	2	1034.29	2.93	0.0866
Treatments	DFF	7	2.57	1.80	0.1647
	PHt (cm)	7	138.73	3.15	**0.0321**
	PrB	7	0.31	2.41	0.0765
	L/P	7	65.25	4.33	**0.0095**
	P/P	7	18.25	3.78	**0.0164**
	PL (cm)	7	0.23	0.51	0.8137
	S/P	7	0.45	0.92	0.5224
	S/PLt	7	2717.20	4.01	**0.0130**
	SW (g)	7	1504.47	17.00	**0.0000**
	SY (g)	7	336.24	0.95	0.4999

Legend: DFF= Days to flowering, **PHt** (cm)= Plant height, **PrB**= Primary branches per plant, **L/P**= Number of leaves or leaflets/plant, **P/P**= Number of pods/plant, **PL** (cm)= Pod length, **S/P**= Number of seeds/pod, **S/PLt**= Number of seeds/plant, **SW**= Seed weight (g), **SY**= Straw yield (g); Significant differences ($p \leq 0.05$) are showed in boldface.

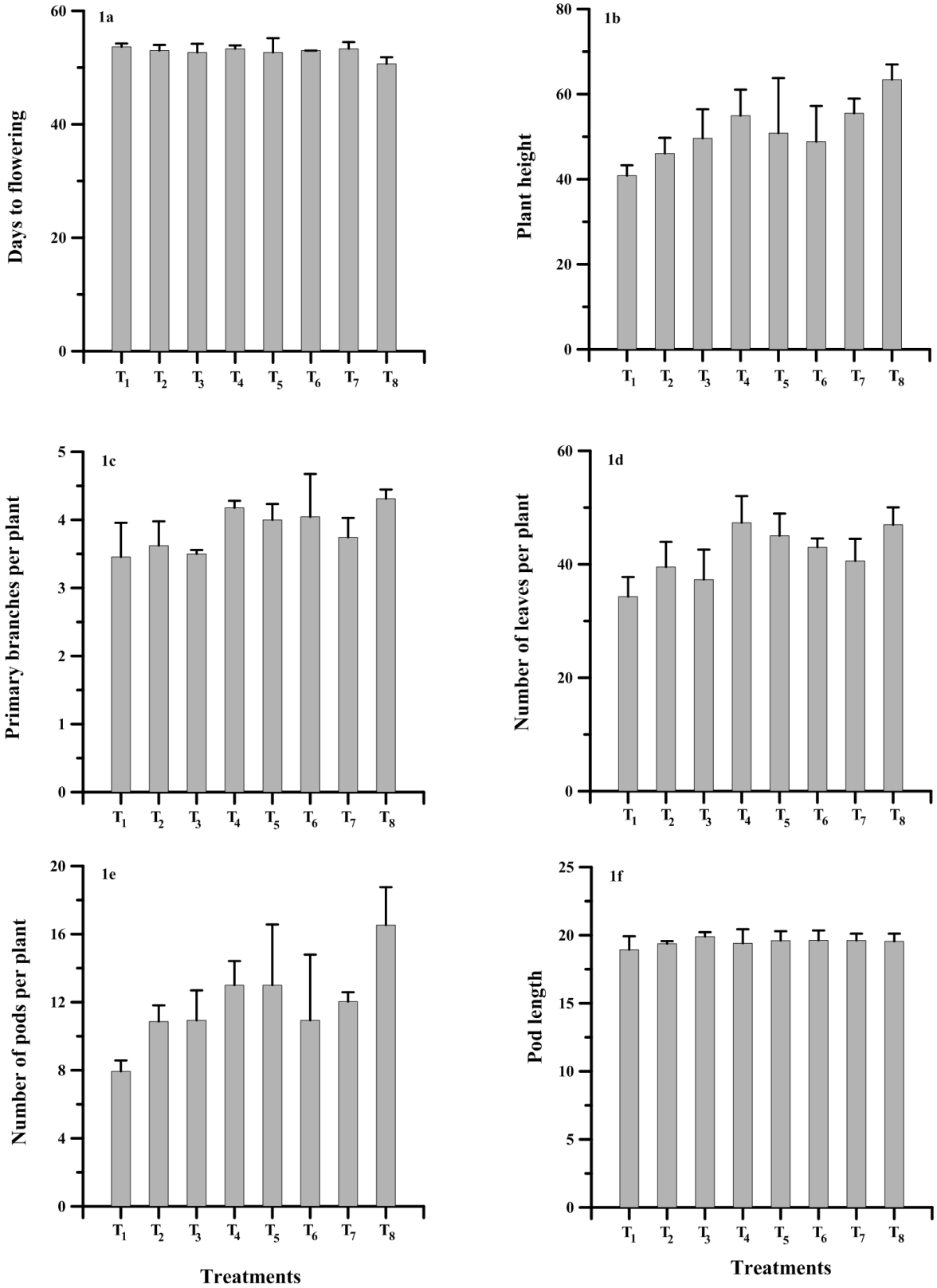
Overall, the nutrient uptake in cowpea legumes is enhanced with NFB and AM fungi inoculation, leading to increased cowpea yield (Omirou *et al*., 2016). It is known that under optimum environmental conditions, different cultivars differ in their morphologies. AM fungi and rhizobia's single and combined application significantly increased seed yield and other growth parameters in *Vicia faba* (Pereira *et al*., 2019).

Table 9.8: Effect of monoxenically produced carrier-based bio-inoculum of *Gi. decipiens*, talc bio-formulation of *Bradyrhizobium* sp. and *Bacillus methylotrophicus* on growth and yield characteristic in *V. unguiculata* differentiated using correlation table.

	DFF	PHt	PrB	L/P	P/P	PL	S/P	S/PLt	SW	SY
DFF	1.00									
PHt	**-0.49**	1.00								
PrB	-0.29	**0.62**	1.00							
L/P	-0.08	**0.63**	**0.73**	1.00						
P/P	**-0.45**	**0.77**	**0.75**	**0.75**	1.00					
PL	0.12	0.02	0.06	0.11	-0.02	1.00				
S/P	0.11	0.00	0.13	0.15	-0.06	**0.63**	1.00			
S/PLt	**-0.42**	**0.76**	**0.75**	**0.76**	**0.97**	0.12	0.18	1.00		
SW	-0.40	**0.76**	**0.57**	**0.68**	**0.86**	0.20	-0.01	**0.84**	1.00	
SY	-0.18	**0.55**	0.39	0.40	**0.64**	-0.03	-0.15	**0.60**	**0.45**	1.00

Legend: DFF= Days to flowering, **PHt** (cm)= Plant height, **PrB**= Primary branches per plant, **L/P**= Number of leaves or leaflets/plant, **P/P**= Number of pods/Plant, **PL** (cm)= Pod Length, **S/P**= Number of seeds/pod, **S/PLt**= Number of seeds/plant, **SW**= Seed weight (g), **SY**= Straw yield (g); Significant correlations are showed in boldface; $p < 0.05$, N=8 (Case-wise deletion of missing data).

In the present study, dual inoculation is effective in cowpea yield improvement. Mirdhe and Lakshman (2014) reported that dual inoculation of AM fungi and rhizobia increased P uptake, nodules number, and root colonization compared to single inoculation. Also, Mathimaran *et al*., (2020) reported that dual inoculation with AM fungi and PGPR in finger millet and pigeon pea cropping system showed increased grain yield than with single inoculation.


1a
Days to flowering
60
40
20
0
1b
Plant height
80
60
40
20
0
1c
Primary branches per plant
5
4
3
2
1
0
1d
Number of leaves per plant
60
40
20
0
1e
Number of pods per plant
20
16
12
8
4
0
1f
Pod length
25
20
15
10
5
0
T1 T2 T3 T4 T5 T6 T7 T8
Treatments

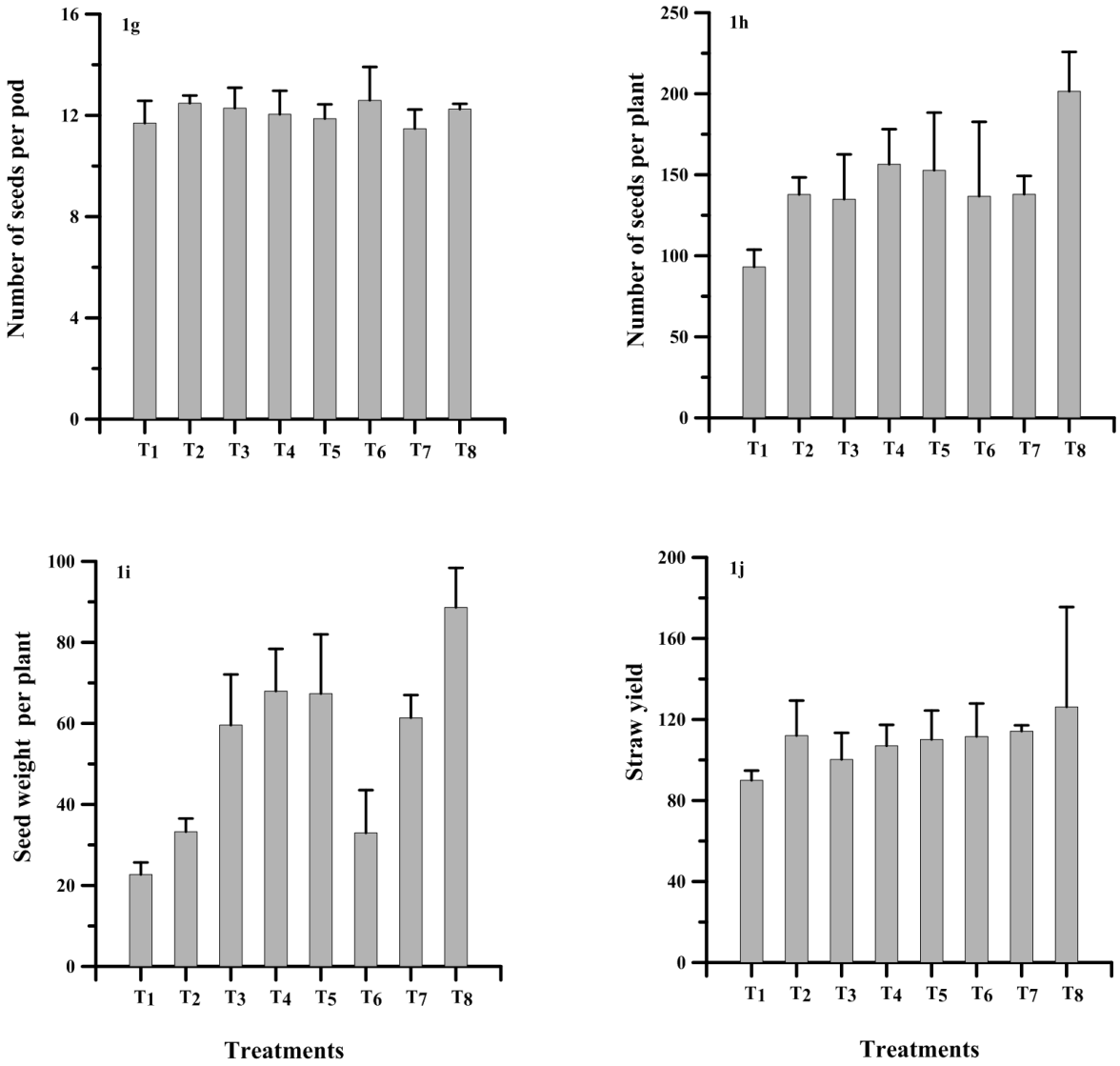


Fig. 9.1: Effect of monoxenically produced carrier-based bio-inoculum of *Gi. decipiens,* bio-formulation of *Bradyrhizobium* sp. and *Bacillus methylotrophicus* on growth and yield characteristic on *V. unguiculata* in experimental field conditions. (1a): Days to flowering, **(1b)**: Plant height, **(1c)**: Primary branches/plant, **(1d)** Number of leaves or leaflets/plant, **(2a):** Number of pods/plant, **(2b):** Pod length, **(2c):** Number of seeds/pod, **(2d):** Number of seeds/plant, **(2e):** Seed weight/plant, **(2f):** Straw yield. All values are the mean of 3 replicates; $\mathbf{T_1}$: Control, $\mathbf{T_2}$: *Gigaspora decipiens*, $\mathbf{T_3}$: *Bradyrhizobium* sp., $\mathbf{T_4}$: *Bacillus methylotrophicus* (Goa Bio-1 culture), $\mathbf{T_5}$: *Gi. decipiens* + *Bradyrhizobium* sp., $\mathbf{T_6}$: *Gi. decipiens* + *B. methylotrophicus*, $\mathbf{T_7}$: *Bradyrhizobium* sp. + *B. methylotrophicus*, $\mathbf{T_8}$: *Gi. decipiens* + *Bradyrhizobium* sp. + *B. methylotrophicus.*

9.4: CONCLUSION

In Goa, even though many varieties and agro-techniques have been developed, the productivity of cowpea has still not reached the desired level. The cowpea plant is mainly grown for dried seeds, fresh leafy vegetables, and straw used for animal feed. The low

productivity of this crop is linked to the low technological level used in its production, low water availability, seeds with low genetic quality, and low use of inputs such as fertilizers and rhizobial inoculants. Tripartite symbiosis *viz.*, legume-rhizobia, and AM fungi association in cowpea significantly improve nodulation, plant growth, nutrient uptake, and N fixation. This leads to improved soil health, increased pod number and weight, and early flowering, increasing yield.

The primary purpose of the present study was to maximize the yield by providing a high number of viable rhizobia in the rhizosphere to allow rapid colonization, nodulation, and N fixation by a selected rhizobial strain. In the present study, inoculation with the indigenous AM fungal species resulted in complementary benefits to the cowpea plant. Inoculation with AM fungi (*Gi. decipiens*) and *Bradyrhizobium* species and *Bacillus methylotrophicus* in cowpea significantly improved yield, thus making the cultivation sustainable.

CHAPTER 10: SUMMARY AND CONCLUSION

The thesis on "Screening for efficient Arbuscular Mycorrhizal (AM) fungal species and Rhizobial strains for enhancing yield in selected local cultivars of *Vigna unguiculata* (L.) Walp (Cowpea)" is an attempt to understand Physico-chemical and biological interaction of the local cultivars of cowpea with reference to AM fungi and Rhizobia. As the Goan cowpea exhibits several superior characteristics owing to its bold size, soft texture, better cooking quality, and exquisite taste, the work examines the possiblity of further enhancing the yield in cowpea through introduction of selected AM fungai and Rhizobial strains.

The Goan cowpea, locally known as *'alsando'* in Goa, is a major pulse crop besides other vegetable crops in the West Coast region. It is an economically important crop and has high nutritional and nutraceutical properties. Further, cowpea improves the soil N levels with no chemical fertilizers due to its unique ability to fix atmospheric N through symbiotic interactions with microbial symbionts such as *Rhizobium*, *Bradyrhizobium*, and AM fungi. This crop has been successfully cultivated on sandy soils. It is tolerant to drought and is extensively grown under residual moisture conditions with a humid climate and temperatures between 29 to 35^0C. The farmers prefer the local cultivar due to its easy availability, better choice of seed material, bold size, and unique taste. However, crop production has not seen the desired yield, although there is an increase in production every year.

The main objective of the present study was to screen and evaluate a promising candidate of AM fungi and rhizobia involved in enhancing the yield of selected local cultivars of *V. unguiculata*. This study involved a detailed survey of cowpea growing areas, identification of various cowpea cultivars from Goa, and studying the Physico-chemical characteristics of cowpea cultivated soils. Further, the abundance and diversity of the AM fungi in different locally available cowpea cultivars, its impact on the enhancement of growth in cowpea, and to standardize the protocol for mass multiplication of AM species using *in vivo* and *in vitro* techniques were assessed. The rhizobial strains associated with cowpea were isolated and identified through molecular methods. Further, a standard carrier for inoculum (AM and *Bradyrhizobium*) was formulated.

In the present study, 18 dominant cowpea growing agricultural sites were studied in Goa. The detailed survey of the various cultivars locally grown in the State and their characteristics resulted in 19 different cultivars, including one local cultivar released by ICAR-CCARI Ella, Old Goa. It was evident that the cultivars differ from place to place in pod length, seed number, size, colour, and yield. A trial field experiment was undertaken at Colva village to compare the yield of 19 cowpea cultivars. Based on the various parameters analyzed, *viz*., pod length, pod dry weight, number of seeds/pod, seed weight/pod, the weight of 100 seeds, and straw yield of pods, the Goa Cowpea-3 (ICAR-CCARI Goa) cultivar showed relatively better growth and yield performance than the other cultivars tested. This cultivar recorded a higher number of pods per plant, seeds per pod, seeds per plant, seed weight per plant, and weight of 100 seeds. Interestingly, the yield data of 50 harvested pods directly from the fields also confirmed that the Goa cowpea-3 cultivar was superior to all the other locally available cultivars.

The Physico-chemical characteristics of the soils revealed that the soil was acidic and non-saline with a pH ranging from 4.7 to 5.9 and containing medium to high Organic Carbon (OC), low to medium levels of Mn, available N, P, and K, and normal levels of Fe, Zn, and Cu. The soils were deficient in DTPA-extractable micronutrients. Acidic pH may be due to the indiscriminate use of inorganic fertilizers and high precipitation in the soil due to Al, Mn, and Fe solubility. Generally, the soils recorded high sand, followed by silt and clay.

The cowpea varieties in Goa vary widely in their phenotypic and genotypic traits and provides better returns for the farmers. Thus a systematic survey of major cowpea growing areas of Goa would identify a superior accession with desirable attributes for large-scale cultivation. One of the significant identified attributes of enhancing yield in cowpea is the appreciable amount of AM fungal diversity in its rhizosphere. However, a detailed study on AM species, soil fertility, fertilizer application, host plant phenology, and competitive abilities of co-occurring AM fungal species is needed to understand the diversity and help develop an efficient bio-inoculum to improve crop productivity in cowpea. To have a holistic understanding of AM fungal diversity of indigenous AM fungal species, the rhizosphere and root samples of the cultivars were analyzed using standard methods. Data was statistically analyzed, and Canonical Correspondence Analysis (CCA) was performed using SPSS 22.0 software package (IBM, Armonk, NY, USA). AM fungal diversity was

highly variable among the sites, and a total of 22 species belonging to 7 genera and three families were identified. The species richness was recorded at maximum at Harmal Pernem and minimum at Porvorim and Mencurem sites.

Gigaspora decipiens was the dominant species, followed by *Gi. margarita*. Further, *Gi. decipiens* ranked highest in spore abundance and relative abundance, indicating its widespread distribution. The presence of intra-radical and extra-radical hyphae, hyphal coils, arbuscules, auxiliary cells, and vesicles was distinct in AM fungal colonization. Maximum root colonization was recorded at Deulwada Harmal, while the minimum was reported at Guirim site. The CCA biplot of AM fungi species *viz.,* A*caulospora delicata, A. spinosa, Scutellospora scutata* revealed a positive correlation with pH, Zn, N, and P at Harmal Pernem and Ozarim-I (S-VIII and S-XIII) study sites.

Another identified attribute involved in enhancing the yield of cowpea is the presence of rhizobial strains associated with the cowpea root nodules. Hence, bacterial isolates associated with cowpea root nodules were isolated. The rhizobial isolates differed significantly in morphological characteristics and colony characters. Based on colony characteristics, two rhizobial strains *viz.,* GUBRS1 and GUBRS2 were isolated. The isolates were Gram-negative and fast-growing on the YEMA medium. These isolates were characterized using molecular techniques at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala. After phylogenetic analysis, obtained sequences were deposited in the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers MH675487 and MH675491.

The sequences of the two rhizobial strains using the NCBI BLASTn tool revealed the greatest sequence similarity with the genus *Bradyrhizobium*. Previous studies have recorded that *Bradyrhizobium* species were the major symbiont of cowpea irrespective of soil type and plant genotype and played a key role in overcoming major N nutrients. Moreover, rhizobial bacteria are tolerant to acidic soil and effectively nodulate cowpea. It is pertinent to note that soil pH in the present study was acidic, and the acidic soil and edaphic factors may be responsible for the diversity of indigenous and novel *Bradyrhizobium* species. Thus, it is suggested that the fast-growing strains may act as candidates for acidic soils and may be employed as commercial inoculants based on adaptation to extreme conditions. The present results further confirm that the isolated

indigenous rhizobial symbionts exhibited nodulation in cowpea, thus indicating their potential to be used in inoculants formulation to achieve maximum legume productivity.

To develop an efficient bio-inoculum for improving the crop productivity in cowpea, it is essential to study the life cycle of AM fungi and the large-scale production of high-quality inocula. Thus, the *in vitro* cultivation of AM fungi is important. Trap and pure cultures were prepared using standard methods. The monoxenic cultures of AM fungi and their long-term experimental study resulted in the successful establishment of effective and efficient inoculum production. Five monospecific cultures obtained from the sampled sites were successfully mass multiplied using coleus (*Plectranthus scutellarioides* (L.) R. Br.) as the host plant. Germination on MSR medium (minus sucrose) was achieved in four AM fungal species *viz., Gi. decipiens, Scutellospora scutata, Racocetra gregaria,* and *Rhizoglomus intraradices. In vitro* colonization was recorded in *S. scutata* and *R. gregaria* with transformed *Linum* roots. *In vitro* spore germination varied from species to species, with *Gi. decipiens* requiring the least number of days.

In vitro colonization and sporulation was successfylly established in *Gi. decipiens* using transformed *Linum* roots on MSR medium. Further, the monoxenic culture produced BAS (Branched absorbing structures) and spores. These two characters are highly desirable while considering an AM fungal strain for mass production of pure and viable inocula. Therefore, the studied indigenous species of *Gi. decipiens* could be produced as monoxenic inocula for biofertilizer inoculum production. Under laboratory conditions, effective mass multiplication was carried out using coleus as the host plant in a phytotron.

The present study isolated nodule forming and N_2 fixing bacteria from *V. unguiculata* (L), Walp, using a YEMA medium. The isolated bacterium was identified as a strain of *Bradyrhizobium*, which grows within 2-4 days' time interval in culture media. As a potential nitrogen-fixing bacteria, talc (CMC as additive) based formulation was prepared and maintained. The application of this bacterial formulation would benefit cowpea farmers in agricultural fields.

However, for mass production of inoculum, optimum carrier materials are required. Bio-formulation was prepared using a mixture of vermiculite: cow dung powder: wood powder: wood ash in the ratio of 20:8:2:1. Results revealed that the materials used for the

formulation of the carrier had different characteristics, *viz.*, cow dung powder had a higher amount of organic carbon (OC), N, P. In contrast, higher potassium (K) was found in wood ash and was alkaline. After the extraction of monoxenically produced propagules of *Gi. decipiens*, the inoculum was prepared in carrier materials using coleus as a host plant. Isolated strains of *Bradyrhizobium* species were grown in YEMB to prepare a talc-based formulation. The study carried out in phytotron reported 100% root colonization in coleus and recorded 130 spores 100 g^{-1}, while the talc-based formulation of *Bradyrhizobium* sp. recorded a concentration of 10^6 CFU mL^{-1}. The bio-formulation thus prepared was successfully packed and stored at room temperature and used as a biofertilizer.

Screening studies using field-based experiments at Colva village in South Goa were carried out to evaluate the effectiveness of *in vitro* produced carrier-based AM bio-inocula, dual inoculation with *Bradyrhizobium* sp., and PGPR on the growth and yield of cowpea plants. The experiment recorded significant root colonization in bio-inoculated treatments compared to un-inoculated control. Further, AM fungal colonization was maximum during the flowering and fruiting stage. Besides, inoculation with bio-inoculants significantly affected the growth and yield characteristics compared to control. Plant height and pod number, seed number, seed yield, and straw yield recorded significant correlation.

Further, tripartite inoculation using *Gi. decipiens*, *Bradyrhizobium* sp., and *B. methylotrophicus* significantly improved yield in cowpea. Therefore, it is inferred that AM fungi, rhizobia, and PGPR could synergistically enhance the yield in cowpea greatly.

CONCLUSION:

In Goa, the cowpea grown is mainly used as dried seed legume, fresh leafy vegetable, and straw for animal feed. However, the desired productivity of cowpea is still not achieved and may be linked to the less usage of advanced technology for agricultural production. The study's primary purpose was to maximize cowpea's yield potential using the indigenously available AM fungi and rhizobia. The following conclusions were drawn from the present study:

1) The present study reveals that the indigenous diversity of AM fungi is low.

2) The indigenous AM fungal species found in the rhizosphere and roots can act as a candidate for acidic soils. They may be employed as commercial bio-inoculant based on adaptation to acidic conditions.

3) Inoculation with the indigenous AM fungal species and novel strains of *Bradyrhizobium* sp. can benefit the cowpea plant.

4) Inoculation with AM fungi (*Gi. decipiens*) together with *Bradyrhizobium* species and *B. methylotrophicus* in cowpea fields grown in fallow rice field conditions may significantly improve yield and make the cultivation sustainable by providing a high number of viable rhizobia to the rhizosphere to allow rapid colonization, nodulation, and N fixation.

REFERENCES:

Abaidoo, R., Dare, M. O., Killani, S., & Opoku, A. (2017). Evaluation of early maturing cowpea (*Vigna unguiculata*) germplasm for variation in phosphorus use efficiency and biological nitrogen fixation potential with indigenous rhizobial populations. *The Journal of Agricultural Science*, *155*(1), 102-116.

Abate, T., Alene, A. D., Bergvinson, D., Silim, S., Orr, A., & Asfaw, S. (2011). Tropical legumes in Africa and South Asia: Knowledge and opportunities. *TL II Research Report No. 1*.

Abd-Alla, M. H., El-Enany, A. W. E., Nafady, N. A., Khalaf, D. M., & Morsy, F. M. (2014). Synergistic interaction of *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (*Vicia faba* L.) in alkaline soil. *Microbiological Research*, *169*(1), 49-58.

Abd-Alla, M. H., Omar, S. A., & Karanxha, S. (2000). The impact of pesticides on arbuscular mycorrhizal and nitrogen-fixing symbioses in legumes. *Applied Soil Ecology*, *14*(3), 191-200.

Abdel-Salam, E., Alatar, A., & El-Sheikh, M. A. (2018). Inoculation with arbuscular mycorrhizal fungi alleviates harmful effects of drought stress on damask rose. *Saudi Journal of Biological Sciences*, *25*(8), 1772-1780.

Adebamowo, C. A., Hu, F. B., Cho, E., Spiegelman, D., Holmes, M. D., & Willett, W. C. (2005). Dietary patterns and the risk of breast cancer. *Annals of Epidemiology*, *15*(10), 789-795.

Adholeya, A. (2003). Commercial production of AMF through industrial mode and its large-scale application. 4th Int. In *Conf. Mycorrhizae. Montreal Quebec Canada* (Vol. 240).

Adholeya, A., Tiwari, P., & Singh, R. (2005). Large-scale inoculum production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. In *In vitro Culture of Mycorrhizas* (pp. 315-338). Springer, Berlin, Heidelberg.

Ahanger, M. A., Tyagi, S. R., Wani, M. R., & Ahmad, P. (2014). Drought tolerance: role of organic osmolytes, growth regulators, and mineral nutrients. *In Physiological Mechanisms and Adaptation Strategies in Plants under Changing Environment* (pp. 25-55). Springer, New York, NY.

Ahmad, F., Uddin, S., Ahmad, N., & Islam, R. (2013). Phosphorus–microbes interaction on growth, yield and phosphorus-use efficiency of irrigated cotton. *Archives of Agronomy and Soil Science*, *59*(3), 341-351.

Ahnia, H., Bourebaba, Y., Duran, D., Boulila, F., Palacios, J. M., Rey, L., & Imperial, J. (2018). *Bradyrhizobium algeriense* sp. nov. a novel species isolated from effective nodules of *Retama sphaerocarpa* from Northeastern Algeria. *Systematic and Applied Microbiology*, *41*(4), 333-339.

Akundabweni, L. S., Peter, P. C., & Singh, B. B. (1990). Evaluation of elite lines of cowpea *(Vigna unguiculata (L.)* Walp.*)* for leaf/fodder plus grain (i.e. dual purpose). *Tropical agriculture*, *67*(2): 133-136.

Alemu, M., Asfaw, Z., Woldu, Z., Fenta, B. A., & Medvecky, B. (2016). Cowpea (*Vigna unguiculata* (L.) Walp.) (Fabaceae) landrace diversity in northern Ethiopia. *International Journal of Biodiversity and Conservation*, *8*(11), 297-309.

Alguacil García, M. D. M., Torres Martínez, M. P., Montesinos-Navarro, A., & Roldán Garrigos, A. (2016). Soil characteristics driving arbuscular mycorrhizal fungal communities in semiarid Mediterranean soils. *Applied and Environmental Microbiology*, *82*(11), 3348-3356.

Almeida, R. T., & Schenck, N. C. (1990). A revision of the genus *Sclerocystis* (Glomaceae, Glomales). *Mycologia*, *82*(6), 703-714.

Amerian, M. R., Stewart, W. S., & Griffiths, H. (2001). Effect of two species of arbuscular mycorrhizal fungi on growth, assimilation and leaf water relations in maize (Zea mays). *Aspects of Applied Biology*, *63*, 71-76.

Ames, R. N., & Linderman, R. G. (1978). The growth of Easter lily (*Lilium longiflorum*) as influenced by vesicular–arbuscular mycorrhizal fungi, *Fusarium oxysporum*, and fertility level. *Canadian Journal of Botany*, *56*(21), 2773-2780.

Ampomah, O. Y., Ofori-Ayeh, E., Solheim, B., & Svenning, M. M. (2008). Host range, symbiotic effectiveness and nodulation competitiveness of some indigenous cowpea bradyrhizobia isolates from the transitional savanna zone of Ghana. *African Journal of Biotechnology*, *7*(8): 988-996.

An, G. H., Kobayashi, S., Enoki, H., Sonobe, K., Muraki, M., Karasawa, T., & Ezawa, T. (2010). How does arbuscular mycorrhizal colonization vary with host plant genotype? An example based on maize (*Zea mays*) germplasms. *Plant and Soil*, *327*(1), 441-453.

Andrade, M. M. M., Stamford, N. P., Santos, C. E. R., Freitas, A. D. S., Sousa, C. A., & Junior, M. A. L. (2013). Effects of biofertilizer with diazotrophic bacteria and mycorrhizal fungi in soil attribute, cowpea nodulation yield and nutrient uptake in field conditions. *Scientia Horticulturae*, *162*, 374-379.

Antunes, P. M., & Goss, M. J. (2005). Communication in the tripartite symbiosis formed by arbuscular mycorrhizal fungi, rhizobia and legume plants: a review. *Roots and Soil Management: Interactions between Roots and the Soil*, *48*, 199-222.

Araújo, A. S. F. D., Carneiro, R. F. V., Bezerra, A. A. C., & Araújo, F. F. D. (2010). Coinoculação rizóbio e *Bacillus subtilis* em feijão-caupi e leucena: efeito sobre a nodulação, a fixação de N_2 e o crescimento das plantas. *Ciência Rural*, *40*(1), 182-185.

Ardakani, S. S., Heydari, A., Khorasani, N., & Arjmandi, R. (2010). Development of new bioformulations of *Pseudomonas fluorescens* and evaluation of these products against damping-off of cotton seedlings. *Journal of Plant Pathology*, *92*, (1), 83-88.

Arora, N. K., Khare, E., & Maheshwari, D. K. (2010). Plant growth promoting rhizobacteria: constraints in bioformulation, commercialization, and future strategies. In *Plant Growth and Health Promoting Bacteria* (pp. 97-116). Springer, Berlin, Heidelberg.

Aseri, G. K., Jain, N., Panwar, J., Rao, A. V., & Meghwal, P. R. (2008). Biofertilizers improve plant growth, fruit yield, nutrition, metabolism and rhizosphere enzyme activities of pomegranate (*Punica granatum* L.) in Indian Thar Desert. *Scientia Horticulturae*, *117*(2), 130-135.

Ashman, M., & Puri, G. (2013). *Essential Soil Science: A Clear and Concise Introduction to Soil Science*. John Wiley & Sons.

Augé, R. M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza*, *11*(1), 3-42.

Avio, L., Pellegrino, E., Bonari, E., & Giovannetti, M. (2006). Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. *New Phytologist*, *172*(2), 347-357.

Ayele, A. (2010). Evaluation of symbiotic effectiveness of rhizobia (*Bradyrhizobium spp*.) with groundnut *(Arachis hypogaea L.)* in Eastern Hararghe zone of Oromiya regional state, Ethiopia (Doctoral dissertation, M. Sc thesis, Haromaya University, Haromaya. 121p).

Azcón, R. (2000). Papel de la simbiosis micorrízica y su interacción con otros microorganismos rizosféricos en el crecimiento vegetal y sostenibilidad agrícola. *Ecología Fisiología y Biotecnología de la Micorriza Arbuscular. Mundi Prensa. México*, 1-15.

Azcón, R., & Barea, J. M. (2010). Mycorrhizosphere interactions for legume improvement. In *Microbes for Legume Improvement* (pp. 237-271). Springer, Vienna.

Azcón-Aguilar, C., & Barea, J. M. (2015). Nutrient cycling in the mycorrhizosphere. *Journal of Soil Science and Plant Nutrition*, *15*(2), 372-396.

Bago, B., & Cano, C. (2005). Breaking myths on arbuscular mycorrhizas in vitro biology. In *In vitro Culture of Mycorrhizas* (pp. 111-138). Springer, Berlin, Heidelberg.

Bago, B., Azcon-Aguilar, C., & Piché, Y. (1998a). Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. *Mycologia*, *90*(1), 52-62.

Bago, B., Azcón-Aguilar, C., Goulet, A., & Piché, Y. (1998b). Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. *The New Phytologist*, *139*(2), 375-388.

Bago, B., Vierheilig, H., Piché, Y., & Azcón-Aguilar, C. (1996). Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. *New Phytologist*, *133*(2), 273-280.

Bainard, L. D., Klironomos, J. N., & Gordon, A. M. (2011). Arbuscular mycorrhizal fungi in tree-based intercropping systems: a review of their abundance and diversity. *Pedobiologia*, *54*(2), 57-61.

Balakrishnan, N., & Subramanian, K. S. (2012). Mycorrhizal symbiosis and bioavailability of micronutrients in maize grain. *Maydica*, *57*(2), 129-138.

Baptista, A., Pinho, O., Pinto, E., Casal, S., Mota, C., & Ferreira, I. M. (2016). Characterization of protein and fat composition of seeds from common beans (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata* L. Walp) and bambara groundnuts (*Vigna subterranea* L. Verdc) from Mozambique. *Journal of Food Measurement and Characterization*, *2*(11), 442-450.

Barrow, J. R., Lucero, M. E., Reyes-Vera, I., & Havstad, K. M. (2008). Do symbiotic microbes have a role in regulating plant performance and response to stress? *Communicative and Integrative Biology*, *1*(1), 69-73.

Bárzana, G., Aroca, R., & Ruiz-Lozano, J. M. (2015). Localized and non-localized effects of arbuscular mycorrhizal symbiosis on accumulation of osmolytes and aquaporins and on antioxidant systems in maize plants subjected to total or partial root drying. *Plant, Cell and Environment*, *38*(8), 1613-1627.

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnology Advances*, *16*(4), 729-770.

Basiru, S., Mwanza, H. P., & Hijri, M. (2021). Analysis of arbuscular mycorrhizal fungal inoculant benchmarks. *Microorganisms*, *9*(1), 81.

Baslam, M., Garmendia, I., & Goicoechea, N. (2011). Arbuscular mycorrhizal fungi (AMF) improved growth and nutritional quality of greenhouse-grown lettuce. *Journal of Agricultural and Food Chemistry*, *59*(10), 5504-5515.

Batista, É. R., Guimarães, S. L., Bonfim-Silva, E. M., & Souza, A. C. P. D. (2017). Combined inoculation of rhizobia on the cowpea development in the soil of Cerrado1. *Revista Ciência Agronômica*, *48*, 745-755.

Bécard, G., & Fortin, J. A. (1988). Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytologist*, *108*(2), 211-218.

Bécard, G., & Piché, Y. (1992). 6 Establishment of Vesicular-arbuscular Mycorrhiza in Root Organ Culture: Review and Proposed Methodology. *Methods in Microbiology*, *24*, 89-108.

Bedini, S., Avio, L., Sbrana, C., Turrini, A., Migliorini, P., Vazzana, C., & Giovannetti, M. (2013). Mycorrhizal activity and diversity in a long-term organic Mediterranean agroecosystem. *Biology and Fertility of Soils*, *49*(7), 781-790.

Bedini, S., Pellegrino, E., Avio, L., Pellegrini, S., Bazzoffi, P., Argese, E., & Giovannetti, M. (2009). Changes in soil aggregation and glomalin-related soil protein content as affected by the arbuscular mycorrhizal fungal species *Glomus mosseae* and *Glomus intraradices*. *Soil Biology and Biochemistry*, *41*(7), 1491-1496.

Beena, K. R., Arun, A. B., Raviraja, N. S., & Sridhar, K. R. (2001). Arbuscular mycorrhizal status of *Polycarpaea corymbosa* (Caryophyllaceae) on sand dunes of West Coast of India. *Ecology, Environment and Conservation*, *7*(4), 355-363.

Beena, K. R., Raviraja, N. S., Arun, A. B., & Sridhar, K. R. (2000c). Diversity of arbuscular mycorrhizal fungi on the coastal sand dunes of the west coast of India. *Current Science*, *79*, 1459-1466.

Begoude, D. A. B., Sarr, P. S., Mpon, T. L. Y., Owona, D. A., Kapeua, M. N., & Araki, S. (2016). Composition of arbuscular mycorrhizal fungi associated with cassava

(*Manihot esculenta* Crantz) cultivars as influenced by chemical fertilization and tillage in Cameroon. *Journal of Applied Biosciences*, *98*, 9270-9283.

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., & Zhang, L. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Frontiers in Plant Science*, *10*, 1068.

Bejandi, T. K., Sharifii, R. S., Sedghi, M., & Namvar, A. (2012). Effects of plant density, *Rhizobium* inoculation and microelements on nodulation, chlorophyll content and yield of chickpea (*Cicer arietinum* L.). *Annals of Biological Research*, *3*(2), 951-958.

Bejarano, A., Ramírez-Bahena, M. H., Velázquez, E., & Peix, A. (2014). *Vigna unguiculata* is nodulated in Spain by endosymbionts of Genisteae legumes and by a new symbiovar (vignae) of the genus *Bradyrhizobium*. *Systematic and applied Microbiology*, *37*(7), 533-540.

Belane, A. K., & Dakora, F. D. (2009). Measurement of N_2 fixation in 30 cowpea (*Vigna unguiculata* L. Walp.) genotypes under field conditions in Ghana, using the ^{15}N natural abundance technique. *Symbiosis*, *48*(1), 47-56.

Belane, A. K., Asiwe, J., & Dakora, F. D. (2011). Assessment of N_2 fixation in 32 cowpea (*Vigna unguiculata* L. Walp) genotypes grown in the field at Taung in South Africa, using ^{15}N natural abundance. *African Journal of Biotechnology*, *10*(55), 11450-11458.

Belay, F., Gebreslasie, A., & Meresa, H. (2017). Agronomic performance evaluation of cowpea [*Vigna unguiculata* (L.) Walp] varieties in Abergelle District, Northern Ethiopia. *Journal of Plant Breeding and Crop Science*, *9*(8), 139-143.

Bell, L. W., Lawrence, J., Johnson, B., & Peoples, M. B. (2017). New ley legumes increase nitrogen fixation and availability and grain crop yields in subtropical cropping systems. *Crop and Pasture Science*, *68*(1), 11-26.

Bennett, A. E., Bever, J. D., & Bowers, M. D. (2009). Arbuscular mycorrhizal fungal species suppress inducible plant responses and alter defensive strategies following herbivory. *Oecologia*, *160*(4), 771-779.

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology*, *84*(1), 11-18.

Berger, K. C., & Truog, E. (1939). Boron determination in soils and plants. *Industrial & Engineering Chemistry Analytical Edition. 11*(10), 540-545.

Berruti, A., Borriello, R., Orgiazzi, A., Barbera, A. C., Lumini, E., & Bianciotto, V. (2014). Arbuscular mycorrhizal fungi and their value for ecosystem management. *Biodiversity: the dynamic balance of the planet. InTech, Rijeta, Croacia*, 159-191.

Bever, J. D., Morton, J. B., Antonovics, J., & Schultz, P. A. (1996). Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *Journal of Ecology*, 71-82.

Bidondo, L. F., Pergola, M., Silvani, V., Colombo, R., Bompadre, J., & Godeas, A. (2012). Continuous and long-term monoxenic culture of the arbuscular mycorrhizal fungus *Gigaspora decipiens* in root organ culture. *Fungal Biology*, *116*(6), 729-735.

Biermann, B., & Linderman, R. G. (1983). Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. *New Phytologist*, *95*(1), 97-105.

Biradar, S., Salimath, P. M., & Patil, B. C. (2010). Land race as a source for improving photosynthetic rate and productivity in cowpea (*Vigna unguiculata* W.). *Electronic Journal of Plant Breeding*, *1*(4), 872-877.

Bivoko, D. R. R., Ahonzo-Niamke, S. L., & Zeze, A. (2013). Impact des propriétés physicochimiques des sols de culture du manioc sur l'abondance et la diversité des communautés de champignons mycorhiziens à arbuscules dans la zone agroécologique d'Azaguie, sud-est de la côte d'ivoire. *Agronomie Africaine*, *25*(3), 251-264.

Błaszkowski, J. (2012). Glomeromycota: W. Szafer Institute of Botany. vol. 23, Kraków: *Polish Academy of Sciences*, pp. 303, ISBN: 978-83-89648-82-2.

Boddington, C. L., & Dodd, J. C. (2000). The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian ultisol. *Plant and Soil*, *218*(1), 137-144.

Bolan, N. S. (1991). A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil*, *134*(2), 189-207.

Bonfante, P., & Anca, I. A. (2009). Plants, mycorrhizal fungi, and bacteria: a network of interactions. *Annual Review of Microbiology*, *63*, 363-383.

Bouchenak, M., & Lamri-Senhadji, M. (2013). Nutritional quality of legumes, and their role in cardiometabolic risk prevention: a review. *Journal of Medicinal Food*, *16*(3), 185-198.

Boukar, O., Coulibaly, O., Fatokun, C., Lopez, K., & Tamò, M. (2012). Innovative research along the cowpea value chain. In *Proceedings of the Fifth World Cowpea Conference on Improving Livelihoods in the Cowpea Value Chain through Advancement in Science. IITA, Nigeria*, pp.56-65.

Boyde, A., & Vesely, P. (1972). Comparison of fixation and drying procedures for preparation of some cultured cell lines for examination in the SEM. *Scanning Electron Microscopy, 1972* (Part II), 265-272.

Bray, R. H., & Kurtz, L. T. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Science*, *59*(1), 39-46.

Broadley, M., Brown, P., Çakmak, İ., Ma, J. F., Rengel, Z. & Zhao, F. (2012). Beneficial elements, In: Marschner, P. (Ed.), Marschner's mineral nutrition of higher plants, Third ed, UK, London, Academic Press, London, pp. 249-269.

Broughton, W. J., & Dilworth, M. (1971). Control of leghaemoglobin synthesis in snake beans. *Biochemical Journal*, *125*(4), 1075-1080.

Brundrett, M. C., & Abbott, L. K. (2002). Arbuscular mycorrhizas in plant communities. In *Microorganisms in Plant Conservation and Biodiversity* (pp. 151-193). Springer, Dordrecht.

Brundrett, M. C., & Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist*, *220*(4), 1108-1115.

Brundrett, M. C., Jasper, D. A., & Ashwath, N. (1999). Glomalean mycorrhizal fungi from tropical Australia II. The effect of nutrient levels and host species on the isolation of fungi. *Mycorrhiza*, *6*(8), 315-321.

Bünger, W., Grönemeyer, J. L., Sarkar, A., & Reinhold-Hurek, B. (2018). *Bradyrhizobium ripae* sp. nov., a nitrogen-fixing symbiont isolated from nodules of wild legumes in Namibia. *International Journal of Systematic and Evolutionary Microbiology*, *68*(12), 3688-3695.

Cao, Y., Wang, E. T., Zhao, L., Chen, W. M., & Wei, G. H. (2014). Diversity and distribution of rhizobia nodulated with *Phaseolus vulgaris* in two ecoregions of China. *Soil Biology and Biochemistry*, *78*, 128-137.

Carlile, M. J., Watkinson, S. C. & Gooday, G. W. (2001). The Fungi Academic Press, San Diego, 588.pp.

Carvalho, L. M., Correia, P. M., Ryel, R. J., & Martins-Loução, M. A. (2003). Spatial variability of arbuscular mycorrhizal fungal spores in two natural plant communities. *Plant and Soil*, *251*(2), 227-236.

Castillo, C. G., Borie, F., Godoy, R., Rubio, R., & Sieverding, E. (2006). Diversity of mycorrhizal plant species and arbuscular mycorrhizal fungi in evergreen forest, deciduous forest and grassland ecosystems of Southern Chile. *Journal of Applied Botany and Food Quality*, *80*(1), 40-47.

Castillo, C. G., Oehl, F., & Sieverding, E. (2016). Arbuscular mycorrhizal fungal diversity in wheat agro-ecosystems in Southern Chile and effects of seed treatment with natural products. *Journal of Soil Science and Plant Nutrition*, *16*(4), 967-978.

Cavagnaro, T. R., Barrios-Masias, F. H., & Jackson, L. E. (2012). Arbuscular mycorrhizas and their role in plant growth, nitrogen interception and soil gas efflux in an organic production system. *Plant and Soil*, *353*(1), 181-194.

Chabot, S., Bécard, G., & Piché, Y. (1992). Life cycle of *Glomus intraradix* in root organ culture. *Mycologia*, *84*(3), 315-321.

Chakraborty, U., Chakraborty, B. N., Chakraborty, A. P., Sunar, K., & Dey, P. L. (2013). Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. *Indian Journal of Biotechnology, 12,* 20–31.

Chianu, J. N., Nkonya, E. M., Mairura, F. S., Chianu, J. N., & Akinnifesi, F. K. (2011). Biological nitrogen fixation and socioeconomic factors for legume production in sub-Saharan Africa: a review. *Agronomy for Sustainable Development*, *31*(1), 139-154.

Chibeba, A. M., Kyei-Boahen, S., de Fátima Guimarães, M., Nogueira, M. A., & Hungria, M. (2017). Isolation, characterization and selection of indigenous *Bradyrhizobium* strains with outstanding symbiotic performance to increase soybean yields in Mozambique. *Agriculture, Ecosystems and Environment*, *246*, 291-305.

Chidebe, I. N., Jaiswal, S. K., & Dakora, F. D. (2018). Distribution and phylogeny of microsymbionts associated with cowpea (*Vigna unguiculata*) nodulation in three agroecological regions of Mozambique. *Applied and Environmental Microbiology*, *84*(2), e01712-17.

Cholin, S., Uma, M. S., Suma, B., & Salimath, P. M. (2010). Stability analysis for yield and yield components over seasons in cowpea [*Vigna unguiculata* L. (Walp.)]. *Electronic Journal of Plant Breeding*, *1*(6), 1392-1395.

Clark, R. Á., & Zeto, S. K. (2000). Mineral acquisition by arbuscular mycorrhizal plants. *Journal of Plant Nutrition*, *23*(7), 867-902.

Coelho, I. R., Pedone-Bonfim, M. V., Silva, F. S., & Maia, L. C. (2014). Optimization of the production of mycorrhizal inoculum on substrate with organic fertilizer. *Brazilian Journal of Microbiology*, *45*, 1173-1178.

Costa, F. A., Haddad, L. S. A. M., Kasuya, M. C. M., Oton, W. C., Costa, M. D., & Borges, A. C. (2013). *In vitro* culture of *Gigaspora decipiens* and *Glomus clarum* in transformed roots of carrot: the influence of temperature and pH. *Acta Scientiarum. Agronomy*, *35*(3), 315-323.

Cranenbrouck, S., Voets, L., Bivort, C., Renard, L., Strullu, D. G., & Declerck, S. (2005). Methodologies for in vitro cultivation of arbuscular mycorrhizal fungi with root organs. In *In vitro Culture of Mycorrhizas* (pp. 341-375). Springer, Berlin, Heidelberg.

Curaqueo, G., Barea, J. M., Acevedo, E., Rubio, R., Cornejo, P., & Borie, F. (2011). Effects of different tillage system on arbuscular mycorrhizal fungal propagules and physical properties in a Mediterranean agroecosystem in central Chile. *Soil and Tillage Research*, *113*(1), 11-18.

Cwala, Y., Laubscher, C. P., Ndakidemi, P. A., & Meyer, A. H. (2010). Mycorrhizal root colonisation and the subsequent host plant response of soilless grown tomato plants in the presence and absence of the mycorrhizal stimulant, Mycotech. *African Journal of Microbiology Research*, *4*(5), 414-419.

da Silva, A. C., da Costa Santos, D., Junior, D. L. T., da Silva, P. B., dos Santos, R. C., & Siviero, A. (2018). Cowpea: A strategic legume species for food security and health. In *Legume Seed Nutraceutical Research*. IntechOpen.

Dabo, M., Jaiswal, S. K., & Dakora, F. D. (2019). Phylogenetic evidence of allopatric speciation of bradyrhizobia nodulating cowpea (*Vigna unguiculata* L. walp) in South African and Mozambican soils. *FEMS Microbiology Ecology*, *95*(6), fiz.067.

Daft, M. J. (1983). The influence of mixed inocula on endomycorrhizal development. In *Tree Root Systems and Their Mycorrhizas* (pp. 331-337). Springer, Dordrecht.

Dakora, F. D., Belane, A. K., Mohale, K. C., Makhubedu, T. I., Makhura, P., Pule-Meulenberg, F., & Oteng-Frimpong, R. (2015). Food grain legumes: their contribution to soil fertility, food security, and human nutrition/health in Africa. *Biological Nitrogen Fixation*, 1063-1070.

Dalpé, Y., & Monreal, M. (2004). Arbuscular mycorrhiza inoculum to support sustainable cropping systems. *Crop Management*, *3*(1), 1-11.

Datta, M., Banik, S., & Gupta, R. K. (1982). Studies on the efficacy of a phytohormone producing phosphate solubilizing *Bacillus firmus* in augmenting paddy yield in acid soils of Nagaland. *Plant and Soil*, *69*(3), 365-373.

Dauber, J., Niechoj, R., Baltruschat, H., & Wolters, V. (2008). Soil engineering ants increase grass root arbuscular mycorrhizal colonization. *Biology and Fertility of Soils*, *44*(5), 791-796.

de Boulois, H. D., Delvaux, B., & Declerck, S. (2005). Effects of arbuscular mycorrhizal fungi on the root uptake and translocation of radiocaesium. *Environmental Pollution*, *134*(3), 515-524.

de Carvalho, F., De Souza, F. A., Carrenho, R., de Souza Moreira, F. M., da Conçeição Jesus, E., & Fernandes, G. W. (2012). The mosaic of habitats in the high-altitude Brazilian rupestrian fields is a hotspot for Arbuscular Mycorrhizal Fungi. *Applied Soil Ecology*, *52*, 9-19.

de Freitas, A. D. S., Silva, A. F., & Sampaio, E. V. D. S. B. (2012). Yield and biological nitrogen fixation of cowpea varieties in the semi-arid region of Brazil. *Biomass and Bioenergy*, *45*, 109-114.

de Santana, A. S., Cavalcante, U. M. T., Sampaio, E. V. D. S. B., & Maia, L. C. (2014). Production, storage and costs of inoculum of arbuscular mycorrhizal fungi (AMF). *Brazilian Journal of Botany*, *37*(2), 159-165.

de Souza Moreira, F. M., Cruz, L., De Faria, S. M., Marsh, T., Martínez-Romero, E., de Oliveira Pedrosa, F., & Young, J. P. W. (2006). *Azorhizobium doebereinerae* sp. nov. microsymbiont of *Sesbania virgata* (Caz.) Pers. *Systematic and Applied Microbiology*, *29*(3), 197-206.

de Souza, F. A., & Declerck, S. (2003). Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. *Mycologia*, *95*(6), 1004-1012.

de Souza, F. A., Dalpé, Y., Declerck, S., de la Providencia, I. E., & Séjalon-Delmas, N. (2005). Life history strategies in Gigasporaceae: insight from monoxenic culture. In *In vitro Culture of Mycorrhizas* (pp. 73-91). Springer, Berlin, Heidelberg.

de Souza, R. G., Goto, B. T., da Silva, D. K. A., da Silva, F. S. B., Sampaio, E. V., & Maia, L. C. (2010). The role of arbuscular mycorrhizal fungi and cattle manure in the establishment of *Tocoyena selloana* Schum. in mined dune areas. *European Journal of Soil Biology*, *46*(3-4), 237-242.

Declerck, S., & Angelo-Van Coppenolle, M. G. (2000). Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. *New Phytologist*, *148*(1), 169-176.

Declerck, S., Dimitri, D. O., Bivort, C., & de Souza, F. A. (2004). Development of extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells. *Mycological Research*, *108*(1), 84-92.

Declerck, S., D'or, D., Cranenbrouck, S., & Boulengé, L. E. (2001). Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. *Mycorrhiza*, *11*(5), 225-230.

Declerck, S., Strullu, D. G., & Fortin, A. (2005). *In vitro culture of mycorrhizas* (Vol. 4). Springer Science & Business Media.

Declerck, S., Strullu, D. G., & Plenchette, C. (1998). Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. *Mycologia*, *90*(4), 579-585.

Degefu, T., Wolde-meskel, E., Ataro, Z., Fikre, A., Amede, T., & Ojiewo, C. O. (2018). Groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* L. Walp) growing in Ethiopia are nodulated by diverse rhizobia. *African Journal of Microbiology Research*, *12*(9), 200-217.

Degefu, T., Wolde-Meskel, E., Woliy, K., & Frostegård, Å. (2017). Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of tree and annual legume species growing in Ethiopia. *Systematic and Applied Microbiology*, *40*(4), 205-214.

Delamuta, J. R. M., Ribeiro, R. A., Menna, P., Bangel, E. V., & Hungria, M. (2012). Multilocus sequence analysis (MLSA) of *Bradyrhizobium* strains: revealing high diversity of tropical diazotrophic symbiotic bacteria. *Brazilian Journal of Microbiology*, *43*, 698-710.

Denton, M. D., Pearce, D. J., & Peoples, M. B. (2013). Nitrogen contributions from faba bean (*Vicia faba* L.) reliant on soil rhizobia or inoculation. *Plant and Soil*, *365*(1), 363-374.

Dessai, S. A., & Rodrigues, B. F. (2012). Diversity studies on arbuscular mycorrhizal fungi in vegetable crop plants of Goa, India. *Plant Pathology and Quarantine*, *2*, 87-101.

Diop, T. (1995). *Ecophysiologie des champignons mycorhiziens à vésicules et arbuscules associés à Acacia albida dans les zones Sahéliennes et Soudano-Guinéenne du Sénégal* (Doctoral dissertation).

Diop, T. A., Bécard, G., & Piché, Y. (1992). Long-term *in vitro* culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. *Symbiosis*. 12: 249-259.

Dioula, B. M., Deret, H., Morel, J., Vachat, E., & Kiaya, V. (2013). Enhancing the role of smallholder farmers in achieving sustainable food and nutrition security. In *International Conference on Nutrition: Better Nutrition Better Lives, Paris*.

Dixon, R. O. D., & Wheeler, C. T. (1986). *Nitrogen fixation in plants.* Blackie and son, Glasgow (UK).

Dodd, J. C., & Jeffries, P. (1986). Early development of vesicular-arbuscular mycorrhizas in autumn-sown cereals. *Soil Biology and Biochemistry*, *18*(2), 149-154.

Douds Jr, D. D., Nagahashi, G., & Hepperly, P. R. (2010). On-farm production of inoculum of indigenous arbuscular mycorrhizal fungi and assessment of diluents of compost for inoculum production. *Bioresource Technology*, *101*(7), 2326-2330.

Douds Jr, D. D., Nagahashi, G., Pfeffer, P. E., Kayser, W. M., & Reider, C. (2005). On-farm production and utilization of arbuscular mycorrhizal fungus inoculum. *Canadian Journal of Plant Science*, *85*(1), 15-21.

Douds Jr, D. D., Nagahashi, G., Pfeffer, P. E., Reider, C., & Kayser, W. M. (2006). On-farm production of AM fungus inoculum in mixtures of compost and vermiculite. *Bioresource Technology*, *97*(6), 809-818.

Douds, D. D., Gadkar, V., & Adholeya, A. (2000). Mass production of VAM fungus biofertilizer. In *Mycorrhizal Biology* (pp. 197-215). Springer, Boston, MA.

Dugje, I. Y., Omoigui, L. O., Ekeleme, F., Kamara, A. Y., & Ajeigbe, H. (2009). Farmers' guide to cowpea production in West Africa. *IITA, Ibadan, Nigeria*, *20*, 12-14.

Dwivedi, S. L., Sahrawat, K. L., Upadhyaya, H. D., Mengoni, A., Galardini, M., Bazzicalupo, M., & Ortiz, R. (2015). Advances in host plant and *Rhizobium* genomics to enhance symbiotic nitrogen fixation in grain legumes. *Advances in Agronomy*, *129*, 1-116.

Dzemo, W. D., Niba, A. S., & Asiwe, J. A. N. (2010). Effects of insecticide spray application on insect pest infestation and yield of cowpea [*Vigna unguiculata* (L.) Walp.] in the Transkei, South Africa. *African Journal of Biotechnology*, *9*(11): 1673-1679.

Echevarría-Zomeño, S., Ariza, D., Jorge, I., Lenz, C., Del Campo, A., Jorrín, J. V., & Navarro, R. M. (2009). Changes in the protein profile of *Quercus ilex* leaves in

response to drought stress and recovery. *Journal of Plant Physiology*, *166*(3), 233-245.

Egamberdieva, D., Reckling, M., & Wirth, S. (2017). Biochar-based *Bradyrhizobium* inoculum improves growth of lupin (*Lupinus angustifolius* L.) under drought stress. *European Journal of Soil Biology*, *78*, 38-42.

El-Hassan, S. A., & Gowen, S. R. (2006). Formulation and delivery of the bacterial antagonist *Bacillus subtilis* for management of lentil vascular wilt caused by *Fusarium oxysporum* f. sp. *lentis*. *Journal of Phytopathology*, *154*(3), 148-155.

Fang, J., Chao, C. C. T., Roberts, P. A., & Ehlers, J. D. (2007). Genetic diversity of cowpea [*Vigna unguiculata* (L.) Walp.] in four West African and USA breeding programs as determined by AFLP analysis. *Genetic Resources and Crop Evolution*, *54*(6), 1197-1209.

Fening, J. O., & Danso, S. K. A. (2002). Variation in symbiotic effectiveness of cowpea bradyrhizobia indigenous to Ghanaian soils. *Applied Soil Ecology*, *21*(1), 23-29.

Figueiredo, M. V., Burity, H. A., Martinez, C. R., & Chanway, C. P. (2008). Alleviation of drought stress in the common bean (*Phaseolus vulgaris* L.) by co-inoculation with *Paenibacillus polymyxa* and *Rhizobium tropici*. *Applied Soil Ecology*, *40*(1), 182-188.

File, A. L., Klironomos, J., Maherali, H., & Dudley, S. A. (2012). Plant kin recognition enhances abundance of symbiotic microbial partner. *Plos one*, *7*(9), p.e45648.

Fitter, A. H. (2006). What is the link between carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. *New Phytologist*, *172*(1), 3-6.

Folk, R. L. (1980). In Petrology of sedimentary rocks. Austin, *Texas*: *Hemphill*. pp. 182.

Folk, R. L., & Lynch, F. L. (1997). The possible role of nanno bacteria (dwarf bacteria) in clay-mineral diagenesis and the importance of careful sample preparation in high-magnification SEM study. *Journal of Sedimentary Research*, *67*(3), 583-589.

Fortin, J. A., Bécard, G., Declerck, S., Dalpé, Y., St-Arnaud, M., Coughlan, A. P., & Piché, Y. (2002). Arbuscular mycorrhiza on root-organ cultures. *Canadian Journal of Botany*, *80*(1), 1-20.

Fortin, J.A., Declerck, S. and Strullu, D. G., (2005). "*In vitro* culture of mycorrhizas", In Soil Biology. *4*, 3-14, Springer, Berlin Heidelberg.

Frank, B. (1885). *Über die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze*. Berichte der Deutschen Botanischen Gesellschaft, *3*, 128-145.

Friesen, T. J., Holloway, G., Hill, G. A., & Pugsley, T. S. (2006). Effect of conditions and protectants on the survival of *Penicillium bilaiae* during storage. *Biocontrol Science and Technology*, *16*(1), 89-98.

Frossard, E., Bünemann, E., Jansa, J., Oberson, A., & Feller, C. (2009). Concepts and practices of nutrient management in agro-ecosystems: Can we draw lessons from history to design future sustainable agricultural production systems. *Die Bodenkultur*, *60*(1), 43-60.

Gaafar, A. E. S. A. (2020). Morphological studies on legume and hilum in seven species of *Vigna savi* (Fabaceae). *African Journal of Plant Science*, *14*(12), 470-480.

Gade, R. M., Chaithanya, B. H., & Khurade, K. C. (2014). A comparative study of different carriers for shelflife of *Pseudomonas fluorescens*. *The Bioscan*, *9*(1), 287-290.

Gadkar, V., Driver, J. D., & Rillig, M. C. (2006). A novel *in vitro* cultivation system to produce and isolate soluble factors released from hyphae of arbuscular mycorrhizal fungi. *Biotechnology Letters*, *28*(14), 1071-1076.

Gamage, H. K., Singhakumara, B. M. P., & Ashton, M. S. (2004). Effects of light and fertilization on arbuscular mycorrhizal colonization and growth of tropical rain-forest *Syzygium* tree seedlings. *Journal of Tropical Ecology*, *20*(5), 525-534.

Gange, A. C., & Smith, A. K. (2005). Arbuscular mycorrhizal fungi influence visitation rates of pollinating insects. *Ecological Entomology*, *30*(5), 600-606.

Gardner, C. M., Laryea, K. B., & Unger, P. W. (1999). *Soil Physical Constraints to Plant Growth and Crop Production* (Vol. 11). Land and Water Development Division, Food and Agriculture Organization.

Garg, N., & Chandel, S. (2011). Arbuscular mycorrhizal networks: process and functions. *Sustainable Agriculture, 2*, 907-930.

Gaur, A., & Adholeya, A. (2000). Effects of the particle size of soil-less substrates upon AM fungus inoculum production. *Mycorrhiza*, *10*(1), 43-48.

Gaur, S., & Purshotam, K. (2011). Influence of Edaphic factors on distribution of mycorrhiza associated with medicinal plants in Indian Central Himalayas. *Journal of Biological Sciences*, *11*(5), 349-358.

Gerdemann, J. W., & Nicolson, T. H. (1963). Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Transactions of the British Mycological Society*, *46*(2), 235-244.

Gianinazzi, S., & Vosátka, M. (2004). Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. *Canadian Journal of Botany*, *82*(8), 1264-1271.

Giller, K. E., Andersson, J. A., Corbeels, M., Kirkegaard, J., Mortensen, D., Erenstein, O., & Vanlauwe, B. (2015). Beyond conservation agriculture. *Frontiers in Plant Science*, *6*, 870.

Gilmore, A. (1968). Phycomycetous mycorrhizal organisms collected by open-pot culture methods. *Hilgardia*, *39*(4), 87-105.

Giovannetti, M., Avio, L., & Sbrana, C. (2010). Fungal spore germination and pre-symbiotic mycelial growth–physiological and genetic aspects. In *Arbuscular Mycorrhizas: Physiology and Function* (pp. 3-32). *Springer*, Dordrecht.

Gitonga, N. M., Koskey, G., Njeru, E. M., Maingi, J. M., & Cheruiyot, R. (2021). Dual inoculation of soybean with *Rhizophagus irregularis* and commercial *Bradyrhizobium japonicum* increases nitrogen fixation and growth in organic and conventional soils. *AIMS Agriculture and Food*, *6*(2): 478–495.

Göhre, V., & Paszkowski, U. (2006). Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. *Planta*, *223*(6), 1115-1122.

Gómez, C. (2004). Cowpea: Post Harvest Operations, Post-harvest Compendium, Food and Agriculture Organization of the United Nations (FAO). Rome, Italy.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., & Srinivas, V. (2016). Formulations of plant growth-promoting microbes for field applications. In *Microbial Inoculants in Sustainable Agricultural Productivity* (pp. 239-251). *Springer*, New Delhi.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L., & Krishnamurthy, L. (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech*, *5*(4), 355-377.

Gosling, P., Hodge, A., Goodlass, G., & Bending, G. D. (2006). Arbuscular mycorrhizal fungi and organic farming. *Agriculture, Ecosystems and Environment*, *113*(1-4), 17-35.

Graham, J. H. (2000). Assessing costs of arbuscular mycorrhizal symbiosis in agroecosystems fungi. *In Current Advances in Mycorrhizae Research*. *APS Press, St. Paul Podila GK Douds Jr DD*, 127-140.

Graham, P. H. (2008). Ecology of the root-nodule bacteria of legumes. In *Nitrogen-fixing leguminous symbioses*, pp. 23-58, *Springer*, Dordrecht.

Grönemeyer, J. L., Kulkarni, A., Berkelmann, D., Hurek, T., & Reinhold-Hurek, B. (2014). Rhizobia indigenous to the Okavango region in Sub-Saharan Africa: diversity, adaptations, and host specificity. *Applied and Environmental Microbiology*, *80*(23), 7244-7257.

Guimarães, A. A., Duque Jaramillo, P. M., Simão Abrahão Nóbrega, R., Florentino, L. A., Barroso Silva, K., & de Souza Moreira, F. M. (2012). Genetic and symbiotic diversity of nitrogen-fixing bacteria isolated from agricultural soils in the western Amazon by using cowpea as the trap plant. *Applied and Environmental Microbiology*, *78*(18), 6726-6733.

Guiñazú, L. B., Andrés, J. A., Del Papa, M. F., Pistorio, M., & Rosas, S. B. (2010). Response of alfalfa (*Medicago sativa* L.) to single and mixed inoculation with phosphate-solubilizing bacteria and *Sinorhizobium meliloti*. *Biology and Fertility of Soils*, *46*(2), 185-190.

Gune, V. T. (1979). Gazetter of the Union Territory of Goa, Daman and Diu, District Gazette Part I Goa. pp. 1-1023.

Güneş, A., Turan, M., Güllüce, M., & Şahin, F. (2014). Nutritional content analysis of plant growth-promoting rhizobacteria species. *European Journal of Soil Biology*, *60*, 88-97.

Gungula, D. T., & Garjila, Y. (2005). The effects of phosphorus application on growth and yield of cowpea in Yola. *Journal of Sustainable Development in Agriculture Environment*, *1*(1), 96-103.

Guo, W., Nazim, H., Liang, Z., & Yang, D. (2016). Magnesium deficiency in plants: An urgent problem. *The Crop Journal*, *4*(2), 83-91.

Habibzadeh, Y. (2015). The effects of arbuscular mycorrhizal fungi and phosphorus levels on dry matter production and root traits in cucumber (*Cucumis sativus* L.). *African Journal of Environmental Science and Technology*, *9*(2), 65-70.

Hale, L., Luth, M., & Crowley, D. (2015). Biochar characteristics relate to its utility as an alternative soil inoculum carrier to peat and vermiculite. *Soil Biology and Biochemistry*, *81*, 228-235.

Hall, A. (2012). Phenotyping cowpeas for adaptation to drought. *Frontiers in Physiology*, *3*, p. 155.

Hanway, J. J., & Heidel, H. (1952). Soil analysis method as used in Iowa State College soil testing laboratory. *Iowa Agriculture*, *57*, 1-31.

Hart, M. M., & Reader, R. J. (2002). Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. *New Phytologist*, *153*(2), 335-344.

Hawkins, H. J., Johansen, A., & George, E. (2000). Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil*, *226*(2), 275-285.

He, F., Sheng, M., & Tang, M. (2017). Effects of *Rhizophagus irregularis* on photosynthesis and antioxidative enzymatic system in *Robinia pseudoacacia* L. under drought stress. *Frontiers in Plant Science*, *8*, p. 183.

Hegde, S. V., & Brahmaprakash, G. P. (1992). A dry granular inoculant of *Rhizobium* for soil application. *Plant and Soil*, *144*(2), 309-311.

Herridge, D. F. (2008). Inoculation technology for legumes. In *Nitrogen-fixing leguminous symbioses* (pp. 77-115). *Springer*, Dordrecht.

Hijri, I., Sýkorová, Z., Oehl, F., Ineichen, K., Mäder, P., Wiemken, A., & Redecker, D. (2006). Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. *Molecular Ecology*, *15*(8), 2277-2289.

Hijri, M. (2016). Analysis of a large dataset of mycorrhiza inoculation field trials on potato shows highly significant increases in yield. *Mycorrhiza*, *26*(3), 209-214.

Hildermann, I., Messmer, M., Dubois, D., Boller, T., Wiemken, A., & Mäder, P. (2010). Nutrient use efficiency and arbuscular mycorrhizal root colonisation of winter wheat cultivars in different farming systems of the DOK long-term trial. *Journal of the Science of Food and Agriculture*, *90*(12), 2027-2038.

Hindumati, A., & Reddy, B. N. (2011). Occurrence and distribution of arbuscular mycorrhizal fungi and microbial flora in the rhizosphere soils of mungbean [*Vigna radiata* (L.) wilczek] and soybean [*Glycine max* (L.) Merr.] from Adilabad, Nizamabad and Karimnagar districts of Andhra Pradesh state, India. *Advances in Bioscience and Biotechnology*, *2*, 275-286.

Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. *Circular. California Agricultural Experiment Station*, *347*(2nd edit).

Hodge, A., Helgason, T., & Fitter, A. H. (2010). Nutritional Ecology of Arbuscular Mycorrhizal Fungi. *Fungal Ecology*, *3*(4), 267-273.

Hoffmann, D., Vierheilig, H., & Schausberger, P. (2011). Mycorrhiza-induced trophic cascade enhances fitness and population growth of an acarine predator. *Oecologia*, *166*(1), 141-149.

Hong, T. D., Edgington, S., Ellis, R. H., de Muro, M. A., & Moore, D. (2005). Saturated salt solutions for humidity control and the survival of dry powder and oil formulations of *Beauveria bassiana* conidia. *Journal of Invertebrate Pathology*, *89*(2), 136-143.

Horn, S., Caruso, T., Verbruggen, E., Rillig, M. C., & Hempel, S. (2014). Arbuscular mycorrhizal fungal communities are phylogenetically clustered at small scales. *The ISME Journal*, *8*(11), 2231-2242.

http://invam.caf.wvu.edu

https://invam.wvu.edu/methods/culture-methods/single-species-cultures

https://invam.wvu.edu/methods/culture-methods/trap-culture

https://www.ncbi.nlm.nih.gov/genbank

IJdo, M., Cranenbrouck, S., & Declerck, S. (2011). Methods for large-scale production of AM fungi: past, present, and future. *Mycorrhiza*, *21*(1), 1-16.

Jackson, M. L. (1973). Soil chemical analysis, Prentice hall of India *Pvt. Ltd., New Delhi, India*, *498*, 151-154.

Jaiswal, S. K., & Dakora, F. D. (2019). Widespread distribution of highly adapted *Bradyrhizobium* species nodulating diverse legumes in Africa. *Frontiers in Microbiology*, *10*, p. 310.

Jaiswal, S. K., Beyan, S. M., & Dakora, F. D. (2016). Distribution, diversity and population composition of soybean-nodulating bradyrhizobia from different agro-climatic regions in Ethiopia. *Biology and Fertility of Soils*, *52*(5), 725-738.

Jakobsen, I. (1985). The role of phosphorus in nitrogen fixation by young pea plants (*Pisum sativum*). *Physiologia Plantarum*, *64*(2), 190-196.

Janoušková, M., Vosátka, M., Rossi, L., & Lugon-Moulin, N. (2007). Effects of arbuscular mycorrhizal inoculation on cadmium accumulation by different tobacco (*Nicotiana tabacum* L.) types. *Applied Soil Ecology*, *35*(3), 502-510.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., & Barea, J. M. (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils*, *37*(1), 1-16.

Ji, B., Bentivenga, S. P., & Casper, B. B. (2012). Comparisons of AM fungal spore communities with the same hosts but different soil chemistries over local and geographic scales. *Oecologia*, *168*(1), 187-197.

Johansson, J. F., Paul, L. R., & Finlay, R. D. (2004). Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiology Ecology*, *48*(1), 1-13.

Johnson, J. M., Houngnandan, P., Kane, A., Chatagnier, O., Sanon, K. B., Neyra, M., & van Tuinen, D. (2016). Colonization and molecular diversity of arbuscular mycorrhizal fungi associated with the rhizosphere of cowpea (*Vigna unguiculata* (L.) Walp.) in Benin (West Africa): an exploratory study. *Annals of Microbiology*, *66*(1), 207-221.

Johnson, J. M., Houngnandan, P., Kane, A., Sanon, K. B., & Neyra, M. (2013). Diversity patterns of indigenous arbuscular mycorrhizal fungi associated with rhizosphere of cowpea (*Vigna unguiculata* (L.) Walp.) in Benin, West Africa. *Pedobiologia*, *56*(3), 121-128.

Johnson, S. E., Lauren, J. G., Welch, R. M., & Duxbury, J. M. (2005). A comparison of the effects of micronutrient seed priming and soil fertilization on the mineral nutrition of chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) in Nepal. *Experimental Agriculture*, *41*(4), 427-448.

Jones, D. L., Nguyen, C., & Finlay, R. D. (2009). Carbon flow in the rhizosphere: carbon trading at the soil–root interface. *Plant and Soil*, *321*(1), 5-33.

Jorjani, M., Heydari, A., Zamanizadeh, H. R., Rezaee, S., & Naraghi, L. (2011). Development of *Pseudomonas fluorescens* and *Bacillus coagulans* based bioformulations using organic and inorganic carriers and evaluation of their influence on growth parameters of sugar beet. *Journal of Biopesticides*, *4*(2), p.180.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., & Pozo, M. J. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *Journal of Chemical Ecology*, *38*(6), 651-664.

Kamara, A. Y., Ewansiha, S., Ajeigbe, H., Omoigui, L., Tofa, A. I., & Karim, K. Y. (2017). Agronomic evaluation of cowpea cultivars developed for the West African Savannas. *Legume Research: An International Journal*, *40*(4), 669-676.

Kandel, P., Sharma, P., Subedi, S., Gupta, S., & Basent, M. (2019). Germplasm evaluation of cowpea (*Vigna unguiculata* (L.) Walp.) in Dang district. *JOJ Wildlife and Biodiversity*, *1*(5), 113-118.

Kandula, J., Stewart, A., & Ridgway, H. J. (2006). Monoxenic culture of the arbuscular mycorrhizal fungus *Scutellospora calospora* and RiTDNA transformed carrot roots. *New Zealand Plant Protection*, *59*, 97-102.

Karandashov, V. E., Kuzovkina, I. N., George, E., & Marschner, H. (1999). Monoxenic culture of arbuscular mycorrhizal fungi and plant hairy roots. *Russian Journal of Plant Physiology*, *46*(1), 87-92.

Karanja, S. M., Kibe, A. M., Karogo, P. N., & Mwangi, M. (2014). Effects of intercrop population density and row orientation on growth and yields of sorghum-cowpea cropping systems in semi-arid rongai, kenya. *Journal of Agricultural Sciences*, *6*, 34-43.

Kebede, E., & Bekeko, Z. (2020). Expounding the production and importance of cowpea (*Vigna unguiculata* (L.) Walp.) in Ethiopia. *Cogent Food and Agriculture*, *6*(1), 1769805.

Khade, S. W., & Adholeya, A. (2007). Feasible bioremediation through arbuscular mycorrhizal fungi imparting heavy metal tolerance: a retrospective. *Bioremediation Journal*, *11*(1), 33-43.

Khade, S. W., & Rodrigues, B. F. (2009). Spore-in-spore syndrome in arbuscular mycorrhizal fungi from agro-based ecosystem of Goa. *Mycorrhiza News,* 21(2): 2-4.

Khalil, S., Loynachan, T. E., & McNabb Jr, H. S. (1992). Colonization of soybean by mycorrhizal fungi and spore populations in Iowa soils. *Agronomy Journal*, *84*(5), 832-836.

Kim, Y. J., Park, H. G., Yang, Y. L., Yoon, Y., Kim, S., & Oh, E. (2005). Multifunctional drug delivery system using starch-alginate beads for controlled release. *Biological and Pharmaceutical Bulletin*, *28*(2), 394-397.

Kimiti, J. M., & Odee, D. W. (2010). Integrated soil fertility management enhances population and effectiveness of indigenous cowpea rhizobia in semi-arid eastern Kenya. *Applied Soil Ecology*, *45*(3), 304-309.

Klironomos, J. N. (2003). Variation in plant response to native and exotic arbuscular mycorrhizal fungi. *Ecology*, *84*(9), 2292-2301.

Klironomos, J. N., & Hart, M. M. (2002). Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. *Mycorrhiza*, *12*(4), 181-184.

Kloepper, J. W., & Schroth, M. N. (1981). Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. *Phytopathology*, *71*(6), 590-592.

Koide, R. T., & Mosse, B. (2004). A history of research on arbuscular mycorrhiza. *Mycorrhiza*, *14*(3), 145-163.

Koide, R. T., Goff, M. D., & Dickie, I. A. (2000). Component growth efficiencies of mycorrhizal and nonmycorrhizal plants. *The New Phytologist*, *148*(1), 163-168.

Koppell, J. H., & Parker, M. A. (2012). Phylogenetic clustering of *Bradyrhizobium* symbionts on legumes indigenous to North America. *Microbiology*, *158*(8), 2050-2059.

Koske, R. E. (1981). *Gigaspora gigantea*: observations on spore germination of a VA-mycorrhizal fungus. *Mycologia*, *73*(2), 288-300.

Koske, R. E., & Tessier, B. (1983). A convenient, permanent slide-mounting medium. *Newsletter of Mycological Society of the America*, 34, 59.

Krasova-Wade, T., Diouf, O., Ndoye, I., Sall, C. E., Braconnier, S., & Neyra, M. (2006). Water-condition effects on rhizobia competition for cowpea nodule occupancy. *African Journal of Biotechnology*, *5*(16): 1457-1463.

Krishnamurthy, K., & Gnanamanickam, S. S. (1998). Biological Control of Rice Blast by *Pseudomonas fluorescens* StrainPf7–14: Evaluation of a Marker Gene and Formulations. *Biological Control*, *13*(3), 158-165.

Kumar, M. V., Lakshmi, G. V., & Madhuvani, P. (2013). Appraisal of soil fertility status in salt-affected soils of Ongole division, Prakasam district, Andhra Pradesh. *Journal of the Indian Society of Soil Science*, *61*(4), 333-340.

Kumar, M., Kaur, A., Pachouri, C. U., & Singh, J. (2015). Growth promoting characteristics of rhizobacteria and AM Fungi for biomass amelioration of *Zea mays*. *Archives of Biological Sciences*, *67*(3), 877-887.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547.

Kumar, V., & Singh, K. P. (2001). Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. *Bioresource Technology*, *76*(2), 173-175.

Kuszala, C., & Gianinazzi-Pearson, V. (2011). Méthodes de conservation hors sol des champignons mycorhizogènes à arbuscules et effets de la dessiccation et de la température sur leur survie. *Cahier des techniques de l'INRA*, *73*, 5-23.

Lanier, J. E., Jordan, D. L., Spears, J. F., Wells, R., & Johnson, P. D. (2005). Peanut response to inoculation and nitrogen fertilizer. *Agronomy Journal*, *97*(1), 79-84.

Laranjo, M., Alexandre, A., & Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiological Research*, *169*(1), 2-17.

Latef, A. A. H. A., & Chaoxing, H. (2014). Does inoculation with *Glomus mosseae* improve salt tolerance in pepper plants? *Journal of Plant Growth Regulation*, *33*(3), 644-653.

Lee, P. J., & Koske, R. E. (1994). *Gigaspora gigantea*: parasitism of spores by fungi and actinomycetes. *Mycological Research*, *98*(4), 458-466.

Lee, Y. J., & George, E. (2005). Development of a nutrient film technique culture system for arbuscular mycorrhizal plants. *HortScience*, *40*(2), 378-380.

Lehmann, A., Veresoglou, S. D., Leifheit, E. F., & Rillig, M. C. (2014). Arbuscular mycorrhizal influence on zinc nutrition in crop plants–a meta-analysis. *Soil Biology and Biochemistry*, *69*, 123-131.

Leite, J., Seido, S. L., Passos, S. R., Xavier, G. R., Rumjanek, N. G., & Martins, L. M. V. (2009). Biodiversity of rhizobia associated with cowpea cultivars in soils of the lower half of the São Francisco River Valley. *Revista Brasileira de Ciência do Solo*, *33*, 1215-1226.

Lekberg, Y. L. V. A., Koide, R. T., Rohr, J. R., Aldrich-Wolfe, L. A. U. R. A., & Morton, J. B. (2007). Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. *Journal of Ecology*, *95*(1), 95-105.

Li, P., Zhang, T., Wang, X., & Yu, D. (2013). Development of biological soil quality indicator system for subtropical China. *Soil and Tillage Research*, *126*, 112-118.

Li, X., Zeng, R., & Liao, H. (2016). Improving crop nutrient efficiency through root architecture modifications. *Journal of Integrative Plant Biology*, *58*(3), 193-202.

Lima, A. S. T. D., Xavier, T. F., Lima, C. E. P. D., Oliveira, J. D. P., Mergulhão, A. C. D. E. S., & Figueiredo, M. D. V. B. (2011). Triple inoculation with *Bradyrhizobium*, *Glomus* and *Paenibacillus* on cowpea (*Vigna unguiculata* [L.] Walp.) development. *Brazilian Journal of Microbiology*, *42*, 919-926.

Lindsay, W. L., & Norvell, W. A. (1978). Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal*, *42*(3), 421-428.

Lira Junior, M. A. (2015). Legume-rhizobia signal exchange: promiscuity and environmental effects. *Frontiers in Microbiology*, *6*, p. 945.

Liu, A., Hamel, C., Hamilton, R. I., Ma, B. L., & Smith, D. L. (2000). Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. *Mycorrhiza*, *9*(6), 331-336.

Liu, W., Zhang, Y., Jiang, S., Deng, Y., Christie, P., Murray, P. J., & Zhang, J. (2016). Arbuscular mycorrhizal fungi in soil and roots respond differently to phosphorus inputs in an intensively managed calcareous agricultural soil. *Scientific Reports*, *6*(1), 1-11.

Lone, R., Shuab, R., Khan, S., Ahmad, J., & Koul, K. K. (2017). Arbuscular mycorrhizal fungi for sustainable agriculture. In *Probiotics and Plant Health* (pp. 553-577). *Springer*, Singapore.

Lovelock, C. E., Wright, S. F., & Nichols, K. A. (2004). Using glomalin as an indicator for arbuscular mycorrhizal hyphal growth: an example from a tropical rain forest soil. *Soil Biology and Biochemistry*, *36*(6), 1009-1012.

Lucy, M., Reed, E., & Glick, B. R. (2004). Applications of free living plant growth-promoting rhizobacteria. *Antonie van leeuwenhoek*, *86*(1), 1-25.

Mabrouk, Y., & Belhadj, O. (2012). Enhancing the biological nitrogen fixation of leguminous crops grown under stressed environments. *African Journal of Biotechnology*, *11*(48), 10809-10815.

Mahajan, G. R., Manjunath, B. L., Latare, A. M., D'Souza, R., Vishwakarma, S., & Singh, N. P. (2016). Microbial and enzyme activities and carbon stock in unique coastal acid saline soils of Goa. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *86*(4), 961-971.

Mahajan, G. R., Manjunath, B. L., Latare, A. M., D'souza, R., Vishwakarma, S., & Singh, N. P. (2015). Fertility status of the unique coastal acid saline soils of Goa. *Journal of the Indian Society of Soil Science*, *63*(2), 232-237.

Mahajan, G., Das, B., Morajkar, S., Desai, A., Murgaokar, D., Kulkarni, R., & Patel, K. (2020). Soil quality assessment of coastal salt-affected acid soils of India. *Environmental Science and Pollution Research*, *27*(21), 26221-26238.

Mahdi, S. S., Hassan, G. I., Samoon, S. A., Rather, H. A., Dar, S. A., & Zehra, B. (2010). Bio-fertilizers in organic agriculture. *Journal of Phytology*, *2*(10), 42-54.

Maia, L. C., & Yano-Melo, A. M. (2001). Germination and germ tube growth of the arbuscular mycorrhizal fungi *Gigaspora albida* in different substrates. *Brazilian Journal of Microbiology*, *32*, 281-285.

Maia, L. C., Silva, F. S. B., & Goto, B. T. (2010). Estrutura, ultraestrutura e germinação de glomerosporos. *Micorrizas*, *30*, 75-116.

Majnoon Hoseini, N. (2008). Grain legume production. Jihad-e-Daneshgahi of Tehran Publishers, pp. 284.

Makarov, M. I. (2019). The role of mycorrhiza in transformation of nitrogen compounds in soil and nitrogen nutrition of plants: a review. *Eurasian Soil Science*, *52*(2), 193-205.

Manjarrez, M., Wallwork, M., Smith, S. E., Smith, F. A., & Dickson, S. (2009). Different arbuscular mycorrhizal fungi induce differences in cellular responses and fungal activity in a mycorrhiza-defective mutant of tomato (rmc). *Functional Plant Biology*, *36*(1), 86-96.

Manjunath, B. L., Ramesh, R., Maruthadurai, R., Desai, H. P., & Singh, N. P. (2013). Technology Package for Higher Productivity and Profitability of Goa Local Cowpea. ICAR Research Complex for Goa (Indian Council of Agricultural Research), Goa, India. *Technical Bulletin, 31*, 1-23.

Manohara, K. K., Morajkar, S., Shanbhag, Y. & Patil, K. (2021). Response of Cowpea [*Vigna unguiculata* (L.) Walp] Genotypes under Residual Moisture Condition in Rice-Fallow Area of Goa State. *Legume Research: An International Journal*, *44*(10), 1138-1143.

Marin, M. (2005). Arbuscular mycorrhizal inoculation in nursery practice. *Handbook of Microbial Biofertilizers*, 289-324, Scottish Agricultural College, Edinburgh, UK.

Marinho, R. D. C. N., Ferreira, L. D. V. M., Silva, A. F. D., Martins, L. M. V., Nóbrega, R. S. A., & Fernandes-Júnior, P. I. (2017). Symbiotic and agronomic efficiency of new cowpea rhizobia from Brazilian Semi-Arid. *Bragantia*, *76*, 273-281.

Martins, L. M. V., Xavier, G. R., Rangel, F. W., Ribeiro, J. R. A., Neves, M. C. P., Morgado, L. B., & Rumjanek, N. G. (2003). Contribution of biological nitrogen fixation to cowpea: a strategy for improving grain yield in the semi-arid region of Brazil. *Biology and Fertility of Soils*, *38*(6), 333-339.

Martins, L. M., Neves, M. C. P., & Rumjanek, N. G. (1997). Growth characteristics and symbiotic efficiency of rhizobia isolated from cowpea nodules of the north-east region of Brazil. *Soil Biology and Biochemistry*, *29*(5-6), 1005-1010.

Mathimaran, N., Jegan, S., Thimmegowda, M. N., Prabavathy, V. R., Yuvaraj, P., Kathiravan, R., & Mäder, P. (2020). Intercropping transplanted pigeon pea with finger millet: Arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria boost yield while reducing fertilizer input. *Frontiers in Sustainable Food Systems*, *4*, 88.

Maun, M. A. (2009). *The Biology of Coastal Sand Dunes*. Oxford University Press.

Mbogne, J. T., Temegne, C. N., Hougnandan, P., Youmbi, E., Tonfack, L. B., & Ntsomboh-Ntsefong, G. (2015). Biodiversity of arbuscular mycorrhizal fungi of pumpkins (*Cucurbita* spp.) under the influence of fertilizers in ferralitic soils of Cameroon and Benin. *Journal of Applied Biology and Biotechnology*, *3*(5), 001-010.

McNear Jr, D. H. (2013). The rhizosphere-roots, soil and everything in between. *Nature Education Knowledge*, *4*(3), 1.

Meding, S. M., & Zasoski, R. J. (2008). Hyphal-mediated transfer of nitrate, arsenic, cesium, rubidium, and strontium between arbuscular mycorrhizal forbs and grasses from a California oak woodland. *Soil Biology and Biochemistry*, *40*(1), 126-134.

Meena, B., Radhajeyalakshmi, R., Marimuthu, T., Vidhyasekaran, P., & Velazhahan, R. (2002). Biological control of groundnut late leaf spot and rust by seed and foliar applications of a powder formulation of *Pseudomonas fluorescens*. *Biocontrol Science and Technology*, *12*(2), 195-204.

Micheni, A., Kihanda, F., & Irungu, J. (2004). Soil organic matter (SOM): The basis for improved crop production in arid and semi-arid climates of eastern Kenya. *Managing nutrient Cycles to Sustain Soil Fertility in Sub-Saharan Africa*, p. 608.

Miller, D. D., Domoto, P. A., & Walker, C. (1985). Mycorrhizal fungi at eighteen apple rootstock plantings in the United States. *New Phytologist*, *100*(3), 379-391.

Millner, P. D., & Kitt, D. G. (1992). The Beltsville method for soilless production of vesicular-arbuscular mycorrhizal fungi. *Mycorrhiza*, *2*(1), 9-15.

Miransari, M. (2010). Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. *Plant Biology*, *12*(4), 563-569.

Miransari, M. (2011). Arbuscular mycorrhizal fungi and nitrogen uptake. *Archives of Microbiology*, *193*(2), 77-81.

Mirdhe, R. M., & Lakshman, H. C. (2014). Synergistic interaction between arbuscular mycorrhizal fungi, *Rhizobium* and phosphate solubilising bacteria on *Vigna unguiculata* (L) Verdc. *International Journal of Bioassays 3*: 2096–2099.

Mohale, K. C., Belane, A. K., & Dakora, F. D. (2014). Symbiotic N nutrition, C assimilation, and plant water use efficiency in Bambara groundnut (*Vigna subterranea* L. Verdc) grown in farmers' fields in South Africa, measured using 15 N and 13 C natural abundance. *Biology and Fertility of Soils*, *50*(2), 307-319.

Mohammed, M., Jaiswal, S. K., & Dakora, F. D. (2018). Distribution and correlation between phylogeny and functional traits of cowpea (*Vigna unguiculata* L. Walp.)-nodulating microsymbionts from Ghana and South Africa. *Scientific Reports*, *8*(1), 1-19.

Mohammed, M., Jaiswal, S. K., & Dakora, F. D. (2019). Insights into the phylogeny, nodule function, and biogeographic distribution of microsymbionts nodulating the orphan Kersting's groundnut [*Macrotyloma geocarpum* (Harms) Marechal & Baudet] in African soils. *Applied and Environmental Microbiology*, *85*(11), e00342-19.

Morton, J. B., & Benny, G. L. (1990). Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon*, *37*, 471-491.

Morton, J. B., Bentivenga, S. P., & Wheeler, W. W. (1993). Germplasm in the International Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) and procedures for culture development, documentation and storage. *Mycotaxon*, *48*(1), 491-528.

Mosse, B. (1959). The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. *Transactions of the British Mycological Society*, *42*(3), 273-286.

Mosse, B., & Hepper, C. (1975). Vesicular-arbuscular mycorrhizal infections in root organ cultures. *Physiological Plant Pathology*, *5*(3), 215-223.

Mpepereki, S., Wollum, A. G., & Makonese, F. (1996). Diversity in symbiotic specificity of cowpea rhizobia indigenous to Zimbabwean soils. *Plant and Soil*, *186*(1), 167-171.

Muchoka, P. J., Njiru, M. D., Nthakanio, N. P., Onyari, C., Kamau, M. P., & Mugendi, N. E. (2020). Arbuscular Mycorrhizal Fungi Colonization in the Rhizosphere of *Aspilia pruliseta* Schweif. ext Schweif in the Semiarid Eastern Kenya. *Air, Soil and Water Research*, *13*, 1178622120969190.

Muchovej, R. M. (2004). Importance of mycorrhizae for agricultural crops. *Gainesville, FL 32611. Institute of Food and Agricultural Sciences (IFAS*), University of Florida. SS-AGR-170.

Mueller, G. M. (2011). *Biodiversity of Fungi: Inventory and Monitoring Methods*. Elsevier, pp, 777.

Mummey, D. L., Antunes, P. M., & Rillig, M. C. (2009). Arbuscular mycorrhizal fungi pre-inoculant identity determines community composition in roots. *Soil Biology and Biochemistry*, *41*(6), 1173-1179.

Mus, F., Crook, M. B., Garcia, K., Garcia Costas, A., Geddes, B. A., Kouri, E. D., & Peters, J. W. (2016). Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Applied and Environmental Microbiology*, *82*(13), 3698-3710.

Muthukumar, T., & Udaiyan, K. (2000). Arbuscular mycorrhizas of plants growing in the Western Ghats region, Southern India. *Mycorrhiza*, *9*(6), 297-313.

Muthukumar, T., & Udaiyan, K. (2002). Seasonality of vesicular-arbuscular mycorrhizae in sedges in a semi-arid tropical grassland. *Acta Oecologica*, *23*(5), 337-347.

Naab, J. B., Chimphango, S. M., & Dakora, F. D. (2009). N_2 fixation in cowpea plants grown in farmers' fields in the Upper West Region of Ghana, measured using ^{15}N natural abundance. *Symbiosis*, *48*(1), 37-46.

Nabiollahi, K., Taghizadeh-Mehrjardi, R., Kerry, R., & Moradian, S. (2017). Assessment of soil quality indices for salt-affected agricultural land in Kurdistan Province, Iran. *Ecological Indicators*, *83*, 482-494.

Nain, L., Yadav, R. C., & Saxena, J. (2012). Characterization of multifaceted *Bacillus* sp. RM-2 for its use as plant growth promoting bioinoculant for crops grown in semi-arid deserts. *Applied Soil Ecology*, *59*, 124-135.

Neumann, E., & George, E. (2005). Extraction of extraradical arbuscular mycorrhizal mycelium from compartments filled with soil and glass beads. *Mycorrhiza*, *15*(7), 533-537.

Newsham, K. K., Upson, R., & Read, D. J. (2009). Mycorrhizas and dark septate root endophytes in polar regions. *Fungal Ecology*, *2*(1), 10-20.

Ngo-Mbogba, M., Yemefack, M., & Nyeck, B. (2015). Assessing soil quality under different land cover types within shifting agriculture in South Cameroon. *Soil and Tillage Research*, *150*, 124-131.

Nielsen, K. B., Kjøller, R., Olsson, P. A., Schweiger, P. F., Andersen, F. Ø., & Rosendahl, S. (2004). Colonisation and molecular diversity of arbuscular mycorrhizal fungi in the aquatic plants *Littorella uniflora* and *Lobelia dortmanna* in southern Sweden. *Mycological Research*, *108*(6), 616-625.

Niklaus, P. A., Alphei, J., Ebersberger, D., Kampichler, C., Kandeler, E., & Tscherko, D. (2003). Six years of *in situ* CO_2 enrichment evoke changes in soil structure and soil biota of nutrient-poor grassland. *Global Change Biology*, *9*(4), 585-600.

Njeru, E. M., Avio, L., Bocci, G., Sbrana, C., Turrini, A., Bàrberi, P., & Oehl, F. (2015). Contrasting effects of cover crops on 'hot spot'arbuscular mycorrhizal fungal communities in organic tomato. *Biology and Fertility of Soils*, *51*(2), 151-166.

Novinscak, A., & Filion, M. (2020). Long Term Comparison of Talc-and Peat-Based Phytobeneficial *Pseudomonas fluorescens and Pseudomonas synxantha* bioformulations for Promoting Plant Growth. *Frontiers in Sustainable Food Systems*, *4*, p. 262.

Nyabyenda, P. (2005). Les plantes cultivées en régions tropicales d'altitude d'Afrique. Les presses *Agronomiques de Gembloux*, p. 253.

Nyaga, J. W., & Njeru, E. M. (2020). Potential of native rhizobia to improve cowpea growth and production in semi-arid regions of Kenya. *Frontiers in Agronomy*, *2*, 28.

Odori, C., Ngaira, J., Kinyua, J., & Nyaboga, E. N. (2020). Morphological, genetic diversity and symbiotic functioning of rhizobia isolates nodulating cowpea (*Vigna unguiculata* L. Walp) in soils of Western Kenya and their tolerance to abiotic stress. *Cogent Food and Agriculture*, *6*(1), 1853009.

O'Hara, G. W. (2001). Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. *Australian Journal of Experimental Agriculture*, *41*(3), 417-433.

Ojha, A., Tak, N., Rathi, S., Chouhan, B., Rao, S. R., Barik, S. K., & Gehlot, H. S. (2017). Molecular characterization of novel *Bradyrhizobium* strains nodulating *Eriosema chinense* and *Flemingia vestita*, important unexplored native legumes of the sub-Himalayan region (Meghalaya) of India. *Systematic and Applied Microbiology*, *40*(6), 334-344.

Okonya, J. S., & Maass, B. L. (2014). Protein and iron composition of cowpea leaves: an evaluation of six cowpea varieties grown in eastern Africa. *African Journal of Food, Agriculture, Nutrition and Development*, *14*(5), 2129-2140.

Oldroyd, G. E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology*, *11*(4), 252-263.

Oldroyd, G. E., Murray, J. D., Poole, P. S. & Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Review of Genetics*, 45, 119-144.

Oliveira, A. N. D., & Oliveira, L. A. D. (2005). Seasonal dynamics of arbuscular mycorrhizal fungi in plants of *Theobroma grandiflorum* Schum and *Paullinia*

cupana Mart. of an agroforestry system in Central Amazonia, Amazonas state, Brazil. *Brazilian Journal of Microbiology*, *36*, 262-270.

Oliveira, R. S., Carvalho, P., Marques, G., Ferreira, L., Pereira, S., Nunes, M., & Freitas, H. (2017). Improved grain yield of cowpea (*Vigna unguiculata*) under water deficit after inoculation with *Bradyrhizobium elkanii* and *Rhizophagus irregularis*. *Crop and Pasture Science*, *68*(11), 1052-1059.

Omirou, M., Fasoula, D. A., & Ioannides, I. M. (2016). *Bradyrhizobium* inoculation alters indigenous AMF community assemblages and interacts positively with AMF inoculum to improve cowpea performance. *Applied Soil Ecology*, *108*, 381-389.

Omomowo, I. O., Fadiji, A. E., & Omomowo, O. I. (2018). Assessment of bio-efficacy of *Glomus versiforme* and *Trichoderma harzianum* in inhibiting powdery mildew disease and enhancing the growth of cowpea. *Annals of Agricultural Sciences*, *63*(1), 9-17.

Omomowo, I. O., Ola, I. O., Akintokun, A. K., Bankole, M. O., & Babalola, O. A. (2009). Direct and residual influence of inoculation with *Glomus mosseae* and *Bradyrhizobium japonicum* on proximate and nutrient element content of cowpea seeds. *American-Eurasian Journal of Sustainable Agriculture*, *3*(3), 435-441.

Öpik, M., & Davison, J. (2016). Uniting species-and community-oriented approaches to understand arbuscular mycorrhizal fungal diversity. *Fungal Ecology*, *24*, 106-113.

Ortas, I. (2010). Effect of mycorrhiza application on plant growth and nutrient uptake in cucumber production under field conditions. *Spanish Journal of Agricultural Research*, (1), 116-122.

Ortaş, I., & Rafique, M. (2017). The mechanisms of nutrient uptake by arbuscular mycorrhizae. In *Mycorrhiza-Nutrient Uptake, Biocontrol, Ecorestoration* (pp. 1-19). *Springer*, Cham.

Ossler, J. N., Zielinski, C. A., & Heath, K. D. (2015). Tripartite mutualism: Facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. *American Journal of Botany*, *102*(8), 1332-1341.

Ossom, E. M., & Rhykerd, R. L. (2007). Effects of corn (*Zea mays* L.) and grain legume associations on soil mineral nutrient concentration, soil temperature, crop yield, land equivalent ratio and gross income in Swaziland. In *8th African Crop Science Society Conference, El-Minia, Egypt, 27-31 October 2007* (pp. 225-230).

Ouallal, I., Abbas, Y., Ech-cheddadi, S., Ouajdi, M., Ouhadach, M., El Yacoubi, H., & Rochdi, A. (2018). Diversité des champignons endomycorhiziens de l'arganier et

potentiel mycorhizogène des sols rhizosphériques des arganeraies du Sud-Ouest marocain. *Bois & Forets Des Tropiques*, *338*, 73-86.

Owen, D., Williams, A. P., Griffith, G. W., & Withers, P. J. (2015). Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. *Applied Soil Ecology*, *86*, 41-54.

Oyewale, R. O., & Bamaiyi, L. J. (2013). Management of cowpea insect pests. *Scholars Academic Journal of Biosciences*, *1*(5), 217-226.

Parker, M. A. (2015). The spread of *Bradyrhizobium* lineages across host legume clades: from Abarema to Zygia. *Microbial Ecology*, *69*(3), 630-640.

Pasquet, R. S. (2000). Allozyme diversity of cultivated cowpea *Vigna unguiculata* (L.) Walp. *Theoretical and Applied Genetics*, *101*(1-2), 211-219.

Pearson, J. N., & Schweiger, P. (1993). *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders associated with subterranean clover: dynamics of colonization, sporulation and soluble carbohydrates. *New Phytologist*, *124*(2), 215-219.

Peksen, A. (2004). Fresh pod yield and some pod characteristics of cowpea (*Vigna unguiculata* L. Walp.) genotypes from Turkey. *Asian Journal of Plant Sciences*, *3*(3), 269-273.

Peoples, M. B., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., & Jensen, E. S. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis*, *48*(1), 1-17.

Peoples, M. B., Herridge, D. F., & Ladha, J. K. (1995). Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production. *Plant Soil*, *174*, 3-28.

Pereira, P. A. A., & Bliss, F. A. (1989). Selection of common bean (*Phaseolus vulgaris* L.) for N_2 fixation at different levels of available phosphorus under field and environmentally-controlled conditions. *Plant and Soil*, *115*(1), 75-82.

Pereira, S., Mucha, Â., Gonçalves, B., Bacelar, E., Látr, A., Ferreira, H., & Marques, G. (2019). Improvement of some growth and yield parameters of faba bean (*Vicia faba*) by inoculation with *Rhizobium laguerreae* and arbuscular mycorrhizal fungi. *Crop and Pasture Science*, *70*(7), 595-605.

Perner, H., Schwarz, D., & George, E. (2006). Effect of mycorrhizal inoculation and compost supply on growth and nutrient uptake of young leek plants grown on peat-based substrates. *HortScience*, *41*(3), 628-632.

Peterson, R. L., Wagg, C., & Pautler, M. (2008). Associations between microfungal endophytes and roots: do structural features indicate function? *Botany*, *86*(5), 445-456.

Phillips, J. M., & Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society*, *55*(1), 158-161.

Picone, C. (2000). Diversity and abundance of arbuscular–mycorrhizal fungus spores in tropical forest and pasture 1. *Biotropica*, *32*, 734-750.

Plenchette, C., & Strullu, D. G. (2003). Long-term viability and infectivity of intraradical forms of *Glomus intraradices* vesicles encapsulated in alginate beads. *Mycological Research*, *107*(5), 614-616.

Plenchette, C., Declerck, S., Diop, T. A., & Strullu, D. G. (1996). Infectivity of monoaxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri-T-DNA-transformed carrot root. *Applied Microbiology and Biotechnology*, *46*(5), 545-548.

Plenchette, C., Fortin, J. A., & Furlan, V. (1983). Growth responses of several plant species to mycorrhizae in a soil of moderate P-fertility. *Plant and Soil*, *70*(2), 199-209.

Pontes, J. S., Oehl, F., Marinho, F., Coyne, D., Silva, D. K. A. D., Yano-Melo, A. M., & Maia, L. C. (2017). Diversity of arbuscular mycorrhizal fungi in Brazil's Caatinga and experimental agroecosystems. *Biotropica*, *49*(3), 413-427.

Powlson, D. S., Prookes, P. C., & Christensen, B. T. (1987). Measurement of soil microbial biomass provides an early indication of changes in total soil organic matter due to straw incorporation. *Soil Biology and Biochemistry*, *19*(2), 159-164.

Prasad, K., Aggarwal, A., Yadav, K., & Tanwar, A. (2012). Impact of different levels of superphosphate using arbuscular mycorrhizal fungi and *Pseudomonas fluorescens* on *Chrysanthemum indicum* L. *Journal of Soil Science and Plant Nutrition*, *12*(3), 451-462.

Prasad, R., Bhola, D., Akdi, K., Cruz, C., Sairam, K. V. S. S., Tuteja, N., & Varma, A. (2017). Introduction to mycorrhiza: historical development. In *Mycorrhiza-Function, Diversity, State of the Art* (pp. 1-7). *Springer*, Cham.

Prell, J., & Poole, P. (2006). Metabolic changes of rhizobia in legume nodules. *Trends in Microbiology*, *14*(4), 161-168.

Pule-Meulenberg, F., Belane, A. K., Krasova-Wade, T., & Dakora, F. D. (2010). Symbiotic functioning and bradyrhizobial biodiversity of cowpea (*Vigna unguiculata* L. Walp.) in Africa. *BMC microbiology*, *10*(1), 1-12.

Puozaa, D. K., Jaiswal, S. K., & Dakora, F. D. (2017). African origin of *Bradyrhizobium* populations nodulating Bambara groundnut (*Vigna subterranea* L. Verdc) in Ghanaian and South African soils. *Plos one*, *12*(9), e0184943.

Puozaa, D. K., Jaiswal, S. K., & Dakora, F. D. (2019). Phylogeny and distribution of *Bradyrhizobium* symbionts nodulating cowpea (*Vigna unguiculata* L. Walp) and their association with the physicochemical properties of acidic African soils. *Systematic and Applied Microbiology*, *42*(3), 403-414.

Rachel, E. K., Reddy, S. R., & Reddy, S. M. (1989). VA mycorrhizal colonization of different angiospermic plant species in the semi-arid soils of Andhra Pradesh. *Acta Botanica Indica*, 17: 225-228.

Radhika, K. P., & Rodrigues, B. F. (2010). Arbuscular mycorrhizal fungal diversity in some commonly occurring medicinal plants of Western Ghats, Goa region. *Journal of Forestry Research*, *21*(1), 45-52.

Radl, V., Simoes-Araujo, J. L., Leite, J., Passos, S. R., Martins, L. M. V., Xavier, G. R., & Zilli, J. E. (2014). *Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil. *International Journal of Systematic and Evolutionary Microbiology*, *64*(3), 725-730.

Raj, B. M., Kumar, R. B., Rao, G. V., & Murthy, K. S. R. (2017). An optimised in vitro protocol for mass production of *Rhizophagus irregularis* spores-for sustainable agriculture. *African Journal of Bacteriology Research*, *9*(4), 21-29.

Ramakrishnan, G., Jeyarajan, R., & Dinakaran, D. (1994). Talc-based formulation of *Trichoderma viride* for biocontrol of *Macrophomina phaseolina*. *Journal of Biological Control*, *8*(1), 41-44.

Ramesh, R., Manjunath, B. L., Maruthadurai, R., Mahajan, G. R., & Singh, N. P. (2013). Cowpea (*Vigna unguiculata*) (Alsando) Production Guidelines.

Rathi, S., Tak, N., Bissa, G., Chouhan, B., Ojha, A., Adhikari, D., & Gehlot, H. S. (2018). Selection of *Bradyrhizobium* or *Ensifer* symbionts by the native Indian caesalpinioid legume *Chamaecrista pumila* depends on soil pH and other edaphic and climatic factors. *FEMS Microbiology Ecology*, *94*(11), fiy180.

Read, D. J., Koucheki, H. K., & Hodgson, J. (1976). Vesicular-arbuscular mycorrhiza in natural vegetation systems. I. The occurrence of infection. *New Phytologist. 77*, 641–653.

Reddy, A. S. (2001). Calcium: silver bullet in signaling. *Plant Science, 160*(3), 381-404.

Reddy, P. P. (2012). Plant growth-promoting rhizobacteria (PGPR). In *Recent Advances In Crop Protection* (pp. 131-158). *Springer*, New Delhi.

Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S. L., Morton, J. B., & Walker, C. (2013). An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). *Mycorrhiza*, *23*(7), 515-531.

Renker, C., Blanke, V., & Buscot, F. (2005). Diversity of arbuscular mycorrhizal fungi in grassland spontaneously developed on area polluted by a fertilizer plant. *Environmental Pollution*, *135*(2), 255-266.

Renner, J. N., Greenlee, L. F., Ayres, K. E., & Herring, A. M. (2015). Electrochemical synthesis of ammonia: a low pressure, low temperature approach. *The Electrochemical Society Interface*, *24*(2), 51-57.

Rillig, M. C., & Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytologist*, *171*(1), 41-53.

Rillig, M. C., Field, C. B., & Allen, M. F. (1999). Soil biota responses to long-term atmospheric CO_2 enrichment in two California annual grasslands. *Oecologia*, *119*(4), 572-577.

Rillig, M. C., Mummey, D. L., Ramsey, P. W., Klironomos, J. N., & Gannon, J. E. (2006). Phylogeny of arbuscular mycorrhizal fungi predicts community composition of symbiosis-associated bacteria. *FEMS Microbiology Ecology*, *57*(3), 389-395.

Rillig, M. C., Wright, S. F., Nichols, K. A., Schmidt, W. F., & Torn, M. S. (2001). Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. *Plant and Soil*, *233*(2), 167-177.

Rodrigues, A. C., Antunes, J. E. L., da Costa, A. F., de Paula Oliveira, J., & Figueiredo, M. D. V. B. (2013). Interrelationship of *Bradyrhizobium* sp. and plant growth-promoting bacteria in cowpea: survival and symbiotic performance. *Journal of Microbiology*, *51*(1), 49-55.

Rodrigues, B. F., & Muthukumar, T. (2009). *Arbuscular Mycorrhizae of Goa: A Manual of Identification Protocols*, Goa University, Goa, 109-135.

Rodrigues, K. M., & Rodrigues, B. F. (2015). Endomycorrhizal association of *Funneliformis mosseae* with transformed roots of *Linum usitatissimum*: germination, colonization, and sporulation studies. *Mycology*, *6*(1), 42-49.

Rodrigues, K. M., & Rodrigues, B. F. (2017). Development of carrier based in vitro produced arbuscular mycorrhizal (AM) fungal inocula for organic agriculture. *Annals of Advanced Agricultural Sciences*, *1*, 26-37.

Rodrigues, K. M., & Rodrigues, B. F. (2018). Maximizing the shelf life of monoxenically produced carrier-based am fungal bio-inocula for maintaining its long-term viability. *Journal of Phytology*. *10*, 43-48.

Roesti, D., Gaur, R., Johri, B. N., Imfeld, G., Sharma, S., Kawaljeet, K., & Aragno, M. (2006). Plant growth stage, fertiliser management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields. *Soil Biology and Biochemistry*, *38*(5), 1111-1120.

Rohr, T. G. (2007). Estudo reológico da mistura carboximetilcelulose e amido e sua utilização como veículo de inoculação bacteriano, p. 98. Dissertação (Mestrado) - Universidade Federal Rural do Rio de Janeiro, Seropédica.

Roth, R., & Paszkowski, U. (2017). Plant carbon nourishment of arbuscular mycorrhizal fungi. *Current Opinion in Plant Biology*, *39*, 50-56.

Rufini, M., Da Silva, M. A. P., Ferreira, P. A. A., de Souza Cassetari, A., Soares, B. L., De Andrade, M. J. B., & de Souza Moreira, F. M. (2014). Symbiotic efficiency and identification of rhizobia that nodulate cowpea in a Rhodic Eutrudox. *Biology and Fertility of Soils*, *50*(1), 115-122.

Ruiz-Lozano, J. M., Collados, C., Barea, J. M., & Azcón, R. (2001). Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants. *New Phytologist*, *151*(2), 493-502.

Rusinamhodzi, L., Murwira, H. K., & Nyamangara, J. (2006). Cotton-cowpea intercropping and its N 2 fixation capacity improves yield of a subsequent maize crop under Zimbabwean rain-fed conditions. *Plant and Soil*, *287*(1), 327-336.

Sadi, M. S., & Masoud, A. (2012). Effect of pH on stability, sunflower growth promotion and biocontrol potential of a talc-based formulation of '*Pseudomonas fluorescens*' UTPF61. *Australian Journal of Crop Science*, *6*(3), 463-469.

Salvagiotti, F., Cassman, K. G., Specht, J. E., Walters, D. T., Weiss, A., & Dobermann, A. (2008). Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. *Field Crops Research*, *108*(1), 1-13.

Sangeetha, D., & Stella, D. (2012). Survival of plant growth promoting bacterial inoculants in different carrier materials. *International Journal of Pharmaceutical and Biological Archives*, *3*(1), 170-178.

Sanz, T., Fernández, M. A., Salvador, A., Munoz, J., & Fiszman, S. M. (2005). Thermogelation properties of methylcellulose (MC) and their effect on a batter formula. *Food Hydrocolloids*, *19*(1), 141-147.

Sardinha, M., Müller, T., Schmeisky, H., & Joergensen, R. G. (2003). Microbial performance in soils along a salinity gradient under acidic conditions. *Applied Soil Ecology*, *23*(3), 237-244.

Sarr, P. S., Yamakawa, T., Saeki, Y., & Guisse, A. (2011). Phylogenetic diversity of indigenous cowpea Bradyrhizobia from soils in Japan based on sequence analysis of the 16S-23S rRNA internal transcribed spacer (ITS) region. *Systematic and Applied Microbiology*, *34*(4), 285-292.

Sarr, P. S.., Fujimoto, S., & Yamakawa, T. (2015). Nodulation, nitrogen fixation and growth of Rhizobia-inoculated cowpea (*Vigna unguiculata* L. Walp) in relation with external nitrogen and light intensity. *International Journal of Plant Biology & Research*, *3*(1), 1025.

Sati, S. C., & Tiwari, M. (2008). *The Mycorrhizae: Diversity, Ecology and Applications*. Daya Books, pp. 359.

Sbrana, C., & Giovannetti, M. (2005). Chemotropism in the arbuscular mycorrhizal fungus *Glomus mosseae*. *Mycorrhiza*, *15*(7), 539-545.

Schenck, N. C. & Perez, Y. (1990). Manual for identification of VA mycorrhizal fungi. INVAM, Florida University, Gainesville, USA, Synergistic Publications.

Scheublin, T. R., Ridgway, K. P., Young, J. P. W., & Van Der Heijden, M. G. (2004). Nonlegumes, legumes, and root nodules harbor different arbuscular mycorrhizal fungal communities. *Applied and Environmental Microbiology*, *70*(10), 6240-6246.

Schreiner, R. P. (2007). Effects of native and nonnative arbuscular mycorrhizal fungi on growth and nutrient uptake of 'Pinot noir' (*Vitis vinifera* L.) in two soils with contrasting levels of phosphorus. *Applied Soil Ecology*, *36*(2-3), 205-215.

Schreiner, R. P., Tarara, J. M., & Smithyman, R. P. (2007). Deficit irrigation promotes arbuscular colonization of fine roots by mycorrhizal fungi in grapevines (*Vitis vinifera* L.) in an arid climate. *Mycorrhiza*, *17*(7), 551-562.

Schultze, M. (2013). Protocols for growing plant symbioses; mycorrhiza. In *Plant Mineral Nutrients* (pp. 47-59). Humana Press, Totowa, NJ.

Schulz, S., Brankatschk, R., Dümig, A., Kögel-Knabner, I., Schloter, M., & Zeyer, J. (2013). The role of microorganisms at different stages of ecosystem development for soil formation. *Biogeosciences*, *10*(6), 3983-3996.

Schüßler, A., & Walker, C. (2010). The Glomeromycota: A Species List With New Families and New Gener. *The Glomeromycota: A species list with new Families and new Gener*a, pp. 58, ISBN 978-1466388048.

Selvaraj, T., & Chellappan, P. (2006). Arbuscular mycorrhizae: a diverse personality. *Journal of Central European Agriculture*, *7*(2), 349-358.

Shafi, M., Bakht, J., Razuddin, Hayat, Y., & Zhang, G. P. (2011). Genotypic difference in the inhibition of photosynthesis and chlorophyll fluorescence by salinity and cadmium stresses in wheat. *Journal of Plant Nutrition*, *34*(3), 315-323.

Shannon, C. E., & Weaver, W. (1949). The Mathematical Theory of Communication. *The University of Illinois Press*, Urbana, USA, pp. 117.

Sharma, K. L., Grace, J. K., Mandal, U. K., Gajbhiye, P. N., Srinivas, K., Korwar, G. R., & Yadav, S. K. (2008). Evaluation of long-term soil management practices using key indicators and soil quality indices in a semi-arid tropical Alfisol. *Soil Research*, *46*(4), 368-377.

Sharma, P., Singh, A., Kahlon, C. S., Brar, A. S., Grover, K. K., Dia, M., & Steiner, R. L. (2018). The role of cover crops towards sustainable soil health and agriculture -A review paper. *American Journal of Plant Sciences*, *9*(9), 1935-1951.

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, *2*(1), 1-14.

Shetty, A. A., Magadum, S., & Managanvi, K. (2013). Vegetables as sources of antioxidants. *Journal of Food and Nutritional Disorders*, *2*(1), 2.

Shockley, F. W., McGraw, R. L., & Garrett, H. E. (2004). Growth and nutrient concentration of two native forage legumes inoculated with *Rhizobium* and Mycorrhiza in Missouri, USA. *Agroforestry Systems*, *60*(2), 137-142.

Siddhuraju, P., & Becker, K. (2007). The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. *Food Chemistry*, *101*(1), 10-19.

Siddique, K. H., Johansen, C., Turner, N. C., Jeuffroy, M. H., Hashem, A., Sakar, D., & Alghamdi, S. S. (2012). Innovations in agronomy for food legumes. A review. *Agronomy for Sustainable Development*, *32*(1), 45-64.

Silva, F. S. B., Yano-Melo, A. M., Brandão, J. A. C., & Maia, L. C. (2005). Sporulation of arbuscular mycorrhizal fungi using Tris-CHl buffer in addition to nutrient solutions. *Brazilian Journal of Microbiology*, *36*(4), 327-332.

Silva, F. V., De Meyer, S. E., Simoes-Araujo, J. L., da Costa Barbe, T., Xavier, G. R., O'Hara, G., & Zilli, J. E. (2014). *Bradyrhizobium manausense* sp. nov., isolated from effective nodules of *Vigna unguiculata* grown in Brazilian Amazonian rainforest soils. *International Journal of Systematic and Evolutionary Microbiology*, *64*(7), 2358-2363.

Silva, F. V., Simões-Araújo, J. L., Silva Júnior, J. P., Xavier, G. R., & Rumjanek, N. G. (2012). Genetic diversity of Rhizobia isolates from Amazon soils using cowpea (*Vigna unguiculata*) as trap plant. *Brazilian Journal of Microbiology*, *43*(2), 682-691.

Simard, S., & Austin, M. (2010). The role of mycorrhizas in forest soil stability with climate change. In *Climate Change and Variability*. IntechOpen. 275.302.

Simpson, E. H. (1949). Measurement of diversity. *Nature*, 163: 688.

Simunji, S., Munyinda, K. L., Lungu, O. I., Mweetwa, A. M., & Phiri, E. (2019). Evaluation of cowpea (*Vigna unguiculata* L. walp) genotypes for biological nitrogen fixation in maize-cowpea crop rotation. *Sustainable Agriculture Research*, *8*(526-2020-510), 82-93.

Singh, A., Parmar, N., Kuhad, R. C., & Ward, O. P. (2011). Bioaugmentation, biostimulation, and biocontrol in soil biology. In *Bioaugmentation, Biostimulation and Biocontrol* (pp. 1-23). *Springer*, Berlin, Heidelberg.

Singh, B. B., Ajeigbe, H. A., Tarawali, S. A., Fernandez-Rivera, S., & Abubakar, M. (2003). Improving the production and utilization of cowpea as food and fodder. *Field Crops Research*, *84*(1-2), 169-177.

Singh, B. B., Ehlers, J. D., Sharma, B., & Freire Filho, F. R. (2002). Recent progress in cowpea breeding. In: Fatokun, C. T., SA; Singh, B. B; Kormawa, P. M. (Ed.),

Challenges and Opportunities for Enhancing Sustainable Cowpea Production. IITA, Ibadan,Nigeria, 22-40.

Singh, S. (2002). Mass production of AM fungi: Part I. *Mycorrhiza News*, *14*(1), 2-10.

Singh, V. (2006). Productivity and economics of rice (*Oryza sativa*)–wheat (*Triticum aestivum*) cropping system under integrated nutrient-supply system in recently reclaimed sodic soil. *Indian Journal of Agronomy*, *51*(2), 81-84.

Sisworo, W. H., Mitrosuhardjo, M. M., Rasjid, H., & Myers, R. J. K. (1990). The relative roles of N fixation, fertilizer, crop residues and soil in supplying N in multiple cropping systems in a humid, tropical upland cropping system. *Plant and Soil*, *121*(1), 73-82.

Smith, S. E., & Read, D. J. (1997). *Mycorrhizal symbiosis*. Academic press. 2nd edition, pp: 605.

Smith, S. E., & Read, D. J. (2008). Mycorrhizal Symbiosis, 3rd edition. New York, London, Burlington, San Diego: *Academic Press*, pp 787.

Smith, S. E., & Read, D. J. (2008a). Mineral nutrition, toxic element accumulation and water relations of arbuscular mycorrhizal plants. *Mycorrhizal Symbiosis*, *3*, 145-148.

Smith, S. E., Jakobsen, I., Grønlund, M., & Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiology*, *156*(3), 1050-1057.

Souchie, E. L., Azcón, R., Barea, J. M., Saggin-Júnior, O. J., & da Silva, E. M. R. (2007). Indolacetic acid production by P-solubilizing microorganisms and interaction with arbuscular mycorrhizal fungi. *Acta Scientiarum. Biological Sciences*, *29*(3), 315-320.

Sousa, C. D. S., Menezes, R. S. C., Sampaio, E. V. D. S. B., Oehl, F., Maia, L. C., Garrido, M. D. S., & Lima, F. D. S. (2012). Occurrence of arbuscular mycorrhizal fungi after organic fertilization in maize, cowpea and cotton intercropping systems. *Acta Scientiarum. Agronomy*, *34*, 149-156.

Srinivasan, R., & Govindasamy, C. (2014). Influence of native arbuscular mycorrhizal fungi on growth, nutrition and phytochemical constituents of *Catharanthus roseus* (L.) G. Don. *Don. Journal of Coastal Life Medicine*, *2*(1), 31-37.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., & Fortin, J. A. (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an *in vitro* system in the absence of host roots. *Mycological Research, 100*(3), 328-332.

Stefan, M., Munteanu, N., Stoleru, V., Mihasan, M., & Hritcu, L. (2013). Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean (*Phaseolus coccineus* L.). *Scientia Horticulturae, 151*, 22-29.

Steinberg, P. D., & Rillig, M. C. (2003). Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. *Soil Biology and Biochemistry, 35*(1), 191-194.

Steinkellner, S., Hage-Ahmed, K., García-Garrido, J. M., Illana, A., Ocampo, J. A., & Vierheilig, H. (2012). A comparison of wild-type, old and modern tomato cultivars in the interaction with the arbuscular mycorrhizal fungus *Glomus mosseae* and the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*. *Mycorrhiza, 22*(3), 189-194.

Strullu, D. G., & Romand, C. (1986). Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. *Comptes rendus de l'Académie des sciences. Série 3, Sciences de la vie, 303*(6), 245-250.

Strullu, D. G., Diop, T., & Plenchette, C. (1997). Réalisation de collections in vitro de *Glomus intraradices* (Schenck et Smith) et *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle de développement. *Comptes Rendus de l'Academie des Sciences Series III Sciences de la Vie, 1*(320), 41-47.

Stürmer, S. L. (2004). Effect of different fungal isolates from the same mycorrhizal community on plant growth and phosphorus uptake in soybean and red clover. *Revista Brasileira de Ciencia do Solo, 28*(4), 611-622.

Subashini, H. D., Malarvannan, S., & Kumaran, P. (2007). Effect of biofertilizers (N-Fixers) on the yield of rice varieties at puducherry, india. *Asian Journal of Agricultural Research, 1*, 146-150.

Subbiah, B. V., & Asija, G. L. (1956). A rapid procedure for the determination of available nitrogen in soils. *Current Science, 25*, 259-260.

Suvorova, A. I., Tjukova, I. S., & Trufanova, E. I. (1999). Thermodynamic and diffusion properties of biodegradable systems based on starch and cellulose derivatives. *Journal of Environmental Polymer Degradation, 7*(1), 35-40.

Sward, R. J. (1981). The structure of the spores of *Gigaspora margarita*. I. The dormant spore. *New Phytologist*, *87*(4), 761-768.

Sylvia, D., Alagely, A., Chellemi, D., & Demchenko, L. (2001). Arbuscular mycorrhizal fungi influence tomato competition with bahiagrass. *Biology and Fertility of Soils*, *34*(6), 448-452.

Tampakaki, A. P., Fotiadis, C. T., Ntatsi, G., & Savvas, D. (2017). Phylogenetic multilocus sequence analysis of indigenous slow-growing rhizobia nodulating cowpea (*Vigna unguiculata* L.) in Greece. *Systematic and Applied Microbiology*, *40*(3), 179-189.

Tawaraya, K. (2003). Arbuscular mycorrhizal dependency of different plant species and cultivars. *Soil Science and Plant Nutrition*, *49*(5), 655-668.

Tchabi, A., Coyne, D., Hountondji, F., Lawouin, L., Wiemken, A., & Oehl, F. (2008). Arbuscular mycorrhizal fungi in the "yam belt". *West Africa African Journal of Biotechnology*, *56*, 256-268.

Tchabi, A., Coyne, D., Hountondji, F., Lawouin, L., Wiemken, A., & Oehl, F. (2010). Efficacy of indigenous arbuscular mycorrhizal fungi for promoting white yam (*Dioscorea rotundata*) growth in West Africa. *Applied Soil Ecology*, *45*(2), 92-100.

Temprano, F., Albareda, M., Camacho, M., Daza, A., Santamaria, C., & Rodríguez-Navarro, N. D. (2002). Survival of several *Rhizobium*/*Bradyrhizobium* strains on different inoculant formulations and inoculated seeds. *International Microbiology*, *5*(2), 81-86.

Thies, J. E., Bohlool, B. B., & Singleton, P. W. (1991). Subgroups of the cowpea miscellany: symbiotic specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*. *Applied and Environmental Microbiology*, *57*(5), 1540-1545.

Thuita, M., Pypers, P., Herrmann, L., Okalebo, R. J., Othieno, C., Muema, E., & Lesueur, D. (2012). Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan soils. *Biology and Fertility of Soils*, *48*(1), 87-96.

Timko, M. P., & Singh, B. B. (2008). Cowpea, a multifunctional legume. In *Genomics of Tropical Crop Plants* (pp. 227-258). *Springer*, New York, NY.

Timko, M. P., Rushton, P. J., Laudeman, T. W., Bokowiec, M. T., Chipumuro, E., Cheung, F., & Chen, X. (2008). Sequencing and analysis of the gene-rich space of cowpea. *BMC genomics*, *9*(1), 1-20.

Tiwari, P., & Adholeya, A. (2002). *In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. *FEMS Microbiology Letters*, *206*(1), 39-43.

Tiwari, P., & Adholeya, A. (2003). Host dependent differential spread of *Glomus intraradices* on various Ri T-DNA transformed roots *in vitro*. *Mycological Progress*, *2*(3), 171-177.

Torrecillas, E., del Mar Alguacil, M., & Roldán, A. (2012). Differences in the AMF diversity in soil and roots between two annual and perennial gramineous plants co-occurring in a Mediterranean, semiarid degraded area. *Plant and Soil*, *354*(1), 97-106.

Trinidad, T. P., Mallillin, A. C., Loyola, A. S., Sagum, R. S., & Encabo, R. R. (2010). The potential health benefits of legumes as a good source of dietary fibre. *British Journal of Nutrition*, *103*(4), 569-574.

Turk, M. A., Assaf, T. A., Hameed, K. M., & Al-Tawaha, A. M. (2006). Significance of mycorrhizae. *World Journal of Agricultural Sciences*, *2*(1), 16-20.

Turrini, A., & Giovannetti, M. (2012). Arbuscular mycorrhizal fungi in national parks, nature reserves and protected areas worldwide: a strategic perspective for their in situ conservation. *Mycorrhiza*, *22*(2), 81-97.

Uchida, R. (2000). Essential nutrients for plant growth: nutrient functions and deficiency symptoms. *Plant Nutrient Management in Hawaii's Soils*, *4*, 31-55.

Unkovich, M., Herridge, D. A. V. I. D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., & Chalk, P. (2008). *Measuring Plant-Associated Nitrogen Fixation in Agricultural Systems*. Australian Centre for International Agricultural Research (ACIAR).

Vafadar, F., Amooaghaie, R., & Otroshy, M. (2014). Effects of plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungus on plant growth, stevioside, NPK, and chlorophyll content of *Stevia rebaudiana*. *Journal of Plant Interactions*, *9*(1), 128-136.

van Der Heijden, M. G., Bakker, R., Verwaal, J., Scheublin, T. R., Rutten, M., Van Logtestijn, R., & Staehelin, C. (2006). Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. *FEMS Microbiology Ecology*, *56*(2), 178-187.

van Der Heijden, M. G., Bardgett, R. D., & Van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, *11*(3), 296-310.

van Der Heijden, M. G., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., & Sanders, I. R. (1998). Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*, *396*(6706), 69-72.

van Veen, J. A., van Overbeek, L. S., & van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews*, *61*(2), 121-135.

Vance, C. P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiology*, *127*(2), 390-397.

Vázquez, M. M., Barea, J. M., & Azcón, R. (2002). Influence of arbuscular mycorrhizae and a genetically modified strain of *Sinorhizobium* on growth, nitrate reductase activity and protein content in shoots and roots of *Medicago sativa* as affected by nitrogen concentrations. *Soil Biology and Biochemistry*, *34*(7), 899-905.

Verbon, E. H., & Liberman, L. M. (2016). Beneficial microbes affect endogenous mechanisms controlling root development. *Trends in Plant Science*, *21*(3), 218-229.

Verma, R., Annapragada, H., Katiyar, N., Shrutika, N., Das, K., & Murugesan, S. (2020). *Rhizobium*. In *Beneficial Microbes in Agro-Ecology* (pp. 37-54). Academic Press.

Vesterager, J. M., Nielsen, N. E., & Høgh-Jensen, H. (2008). Effects of cropping history and phosphorus source on yield and nitrogen fixation in sole and intercropped cowpea–maize systems. *Nutrient Cycling in Agroecosystems*, *80*(1), 61-73.

Vidhyasekaran, P., & Muthamilan, M. (1995). Development of formulations of *Pseudomonas fluorescens* for control of chickpea wilt. *Plant Disease (USA)*, *79*, 780-782.

Vidhyasekaran, P., Sethuraman, K., Rajappan, K., & Vasumathi, K. (1997). Powder Formulations of *Pseudomonas fluorescensto* Control Pigeon pea Wilt. *Biological Control*, *8*(3), 166-171.

Vieira, R. F., Mendes, I. C., Reis-Junior, F. B., & Hungria, M. (2010). Symbiotic nitrogen fixation in tropical food grain legumes: current status. In *Microbes for Legume Improvement* (pp. 427-472). *Springer*, Vienna.

Vincent, J. M. (1970). A manual for the practical study of the root-nodule bacteria, International Biological Program. Blackwell Scientific Publications, Oxford. *15*, 164.

Voets, L., Goubau, I., Olsson, P. A., Merckx, R., & Declerck, S. (2008). Absence of carbon transfer between *Medicago truncatula* plants linked by a mycorrhizal network, demonstrated in an experimental microcosm. *FEMS Microbiology Ecology*, *65*(2), 350-360.

Wade, T. K., Le Quéré, A., Laguerre, G., N'Zoué, A., Ndione, J. A., Sadio, O., & Neyra, M. (2014). Eco-geographical diversity of cowpea bradyrhizobia in Senegal is marked by dominance of two genetic types. *Systematic and Applied Microbiology*, *37*(2), 129-139.

Wagner, S. C. (2011). Biological nitrogen fixation. *Nature Education Knowledge*, *3*(10), 15.

Walker, C., & Vestberg, M. (1994). A simple and inexpensive method for producing and maintaining closed pot cultures of arbuscular mycorrhizal fungi. *Agricultural and Food Science*, *3*(3), 233-240.

Walker, T. (2016). *Improved crop productivity for Africa's Drylands*. World Bank Publications, Washington, DC: World Bank. doi: 10.1596/978-1-4648-0896-8.

Walkley, A. J., & Black, I. A. (1934). An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Science. 37,* 29-38.

Wang, B., & Qiu, Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza*, *16*(5), 299-363.

Wang, L., Cao, Y., Wang, E. T., Qiao, Y. J., Jiao, S., Liu, Z. S., & Wei, G. H. (2016). Biodiversity and biogeography of rhizobia associated with common bean (*Phaseolus vulgaris* L.) in Shaanxi Province. *Systematic and Applied Microbiology*, *39*(3), 211-219.

Wang, W. K. (2004). *U.S. Patent No. 6,759,232*. Washington, DC: U.S. Patent and Trademark Office.

Weisany, W., Raei, Y., & Allahverdipoor, K. H. (2013). Role of some of mineral nutrients in biological nitrogen fixation. *Bulletin of Environment, Pharmacology and Life Sciences*, *2*(4), 77-84.

Willis, A., Rodrigues, B. F., & Harris, P. J. (2013). The ecology of arbuscular mycorrhizal fungi. *Critical Reviews in Plant Sciences*, *32*(1), 1-20.

Wiseman, P. E.., Colvin, K. H., & Wells, C. E. (2009). Performance of mycorrhizal products marketed for woody landscape plants. *Journal of Environmental Horticulture*, *27*(1), 41-50.

Wright, S. F., & Upadhyaya, A. (1998). A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant and Soil*, *198*(1), 97-107.

Wubet, T., Weiß, M., Kottke, I., Teketay, D., & Oberwinkler, F. (2004). Molecular diversity of arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia. *New Phytologist*, *161*(2), 517-528.

Xu, G., Fan, X., & Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology*, *63*, 153-182.

Yadav, J., & Verma, J. P. (2014). Effect of seed inoculation with indigenous *Rhizobium* and plant growth promoting rhizobacteria on nutrients uptake and yields of chickpea (*Cicer arietinum* L.). *European Journal of Soil Biology*, *63*, 70-77.

Yánez-Mendizabal, V., Viñas, I., Usall, J., Cañamás, T., & Teixidó, N. (2012). Endospore production allows using spray-drying as a possible formulation system of the biocontrol agent *Bacillus subtilis* CPA-8. *Biotechnology Letters*, *34*(4), 729-735.

Yano, K., & Takaki, M. (2005). Mycorrhizal alleviation of acid soil stress in the sweet potato (*Ipomoea batatas*). *Soil Biology and Biochemistry*, *37*(8), 1569-1572.

Yardin, M. R., Kennedy, I. R., & Thies, J. E. (2000). Development of high quality carrier materials for field delivery of key microorganisms used as bio-fertilisers and bio-pesticides. *Radiation Physics and Chemistry*, *57*(3-6), 565-568.

Yaseen, T., Burni, T., & Hussain, F. (2011). Effect of arbuscular mycorrhizal inoculation on nutrient uptake, growth and productivity of cowpea (*Vigna unguiculata*) varieties. *African Journal of Biotechnology*, *10*(43), 8593-8598.

Yoseph, T., Baraso, B., & Ayalew, T. (2017). Influence of Bradyrhizobia inoculation on growth, nodulation and yield performance of cowpea varieties. *African Journal of Agricultural Research*, *12*(22), 1906-1913.

Youseif, S. H., El-Megeed, A., Fayrouz, H., & Saleh, S. A. (2017). Improvement of faba bean yield using *Rhizobium*/*Agrobacterium* inoculant in low-fertility sandy soil. *Agronomy*, *7*(1), 2.

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews*, *63*(4), 968-989.

Zhang, Y. F., Wang, E. T., Tian, C. F., Wang, F. Q., Han, L. L., Chen, W. F., & Chen, W. X. (2008). *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and

Bradyrhizobium japonicum are the main rhizobia associated with *Vigna unguiculata* and *Vigna radiata* in the subtropical region of China. *FEMS Microbiology Letters*, *285*(2), 146-154.

Zhang, Y. M., Li Jr, Y., Chen, W. F., Wang, E. T., Tian, C. F., Li, Q. Q., & Chen, W. X. (2011). Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain. *Applied and Environmental Microbiology*, *77*(18), 6331-6342.

Zhu, X. C., Song, F. B., Liu, S. Q., Liu, T. D., & Zhou, X. (2012). Arbuscular mycorrhizae improves photosynthesis and water status of *Zea mays* L. under drought stress. *Plant, Soil and Environment*, *58*(4), 186-191.

Zhu, Y. G., Smith, A. F., & Smith, S. E. (2003). Phosphorus efficiencies and responses of barley (*Hordeum vulgare* L.) to arbuscular mycorrhizal fungi grown in highly calcareous soil. *Mycorrhiza*, *13*(2), 93-100.

Zilli, J. É., Valicheski, R. R., Rumjanek, N. G., Simões-Araújo, J. L., Freire Filho, F. R., & Neves, M. C. P. (2006). Symbiotic efficiency of cowpea *Bradyrhizobium* strains in Cerrado soils. *Pesquisa Agropecuária Brasileira*, *41*(5), 811-818.

Zilli, J. É., Valisheski, R. R., Freire Filho, F. R., Neves, M. C. P., & Rumjanek, N. G. (2004). Assessment of cowpea rhizobium diversity in Cerrado areas of northeastern Brazil. *Brazilian Journal of Microbiology*, *35*, 281-287.

Zinga, M. K., Jaiswal, S. K., & Dakora, F. D. (2017). Presence of diverse rhizobial communities responsible for nodulation of common bean (*Phaseolus vulgaris*) in South African and Mozambican soils. *FEMS Microbiology Ecology*, *93*(2), 236.

Publication:

- **Velip, D. M.** and Rodrigues, B. F. (2019). *In vitro* cultivation of *Gigaspora decipiens* using transformed roots of *Linum usitatissimum*. *Kavaka*, 53: pp. 96-99.

Presentations at Conferences:

1. **Velip, D. M.** and Rodrigues, B. F. (2015). Arbuscular Mycorrhizal (AM) Fungal Diversity in local cultivars of *Vigna unguiculata* (L.) Walp (Cowpea) from Goa region. In: Asian Mycological Congress. Co-organized by Department of Botany, Goa University, Goa, Asian Mycological Association (AMA) and Mycological Society of India (MSI) from 7th-10th October 2015 (Poster).

2. **Velip, D. M.** and Rodrigues, B. F. (2017). *In vitro* culture of Arbuscular Mycorrhizal (AM) Fungi associated with *Vigna unguiculata* (L.) Walp (Cowpea) from Goa. In: National conference on Reaching the unreached through science and technology: recent advances in physical, chemical, mathematical and biological sciences for energy, health and environment organized by Mangalore University from 8th-9th September 2017 (Poster).

3. **Velip, D. M.** and Rodrigues, B. F. (2020). Novel strains of *Bradyrhizobium* sp. isolated from root nodules of *Vigna unguiculata* (L.) Walp (Cowpea) fields of Goa. In: National conference on New Vistas in Botany, organized by UGC-SAP and Department of Botany, Goa University, Goa from 13-14th February 2020 (Poster).

4. **Velip, D. M.** and Rodrigues, B. F. (2021). Arbuscular Mycorrhizal fungal diversity in *Vigna unguiculata* from agricultural fields of Goa. In: National Conference on Biodiversity and Biotechnology of Fungi. Co-organized by Department of Botany, Punjabi University, Patiala from 22nd – 24th February 2021 (Poster).

Plate 3.1: Google earth reference images used to identify *V. unguiculata* cultivation in the agricultural fields of Goa state. **a:** Sangolda-I (S-I), **b:** Sangolda-II (S-II), **c:** Sangolda -III (S-III), **d:** Macasana (S-IV), **e:** Moira (S-V), **f:** Dadachiwadi Dhargal (S-VI).

Plate 3.2: Google earth reference images used to identify *V. unguiculata* cultivation in the agricultural fields of Goa state. **a:** Deulwada Harmal (S-VII), **b:** Harmal Pernem (S-VIII), **c:** Corjuem (S-IX), **d:** Coimavaddo Aldona (S-X), **e:** Utorda (S-XI), **f:** Mencurem (S-XII).

Plate 3.3: Google earth reference images used to identify *V. unguiculata* cultivation in the agricultural fields of Goa state. **a:** Ozarim-I (S-XIII), **b:** Ozarim-II (S-XIV), **c:** Dulape (S-XV), **d:** Colva (S-XVI), **e:** Porvorim (S-XVII), **f:** Guirim (S-XVIII).

Plate 3.4: Cultivation of selected cowpea cultivars in the field. **a:** Field preparation, **b-c:** Mulching, **d:** Plants growing in the field.

Plate 3.5: Cowpea cultivars grown in field. **a:** Sangolda-I= Goa SA-1, **b:** Sangolda-II= Goa SA-2, **c:** Sangolda-III= Goa SA-3, **d:** Macasana= Goa MA-1, **e:** Moira= Goa MO-1, **f:** Dadachiwadi Dhargalim= Goa DD-1.

Plate 3.6: Cowpea cultivars grown in field. **a:** Deulwada Harmal= Goa DH-1, **b:** Harmal Pernem= Goa HA-1, **c:** Corjuem= Goa COR-1, **d:** Coimavaddo Aldona= Goa COI-1, **e:** Utorda= Goa UT-1, **f:** Mencurem= Goa ME-1.

Plate 3.7: Cowpea cultivars grown in field. **a:** Ozarim-I= Goa OZ-1, **b:** Ozarim-II = Goa OZ-2, **c:** Dulape= Goa DU-1, **d:** Colva= Goa COL-1, **e:** Porvorim= Goa PO-1, **f:** Guirim= Goa GU-1, **g:** Goa cowpea-3= Goa CO-3.

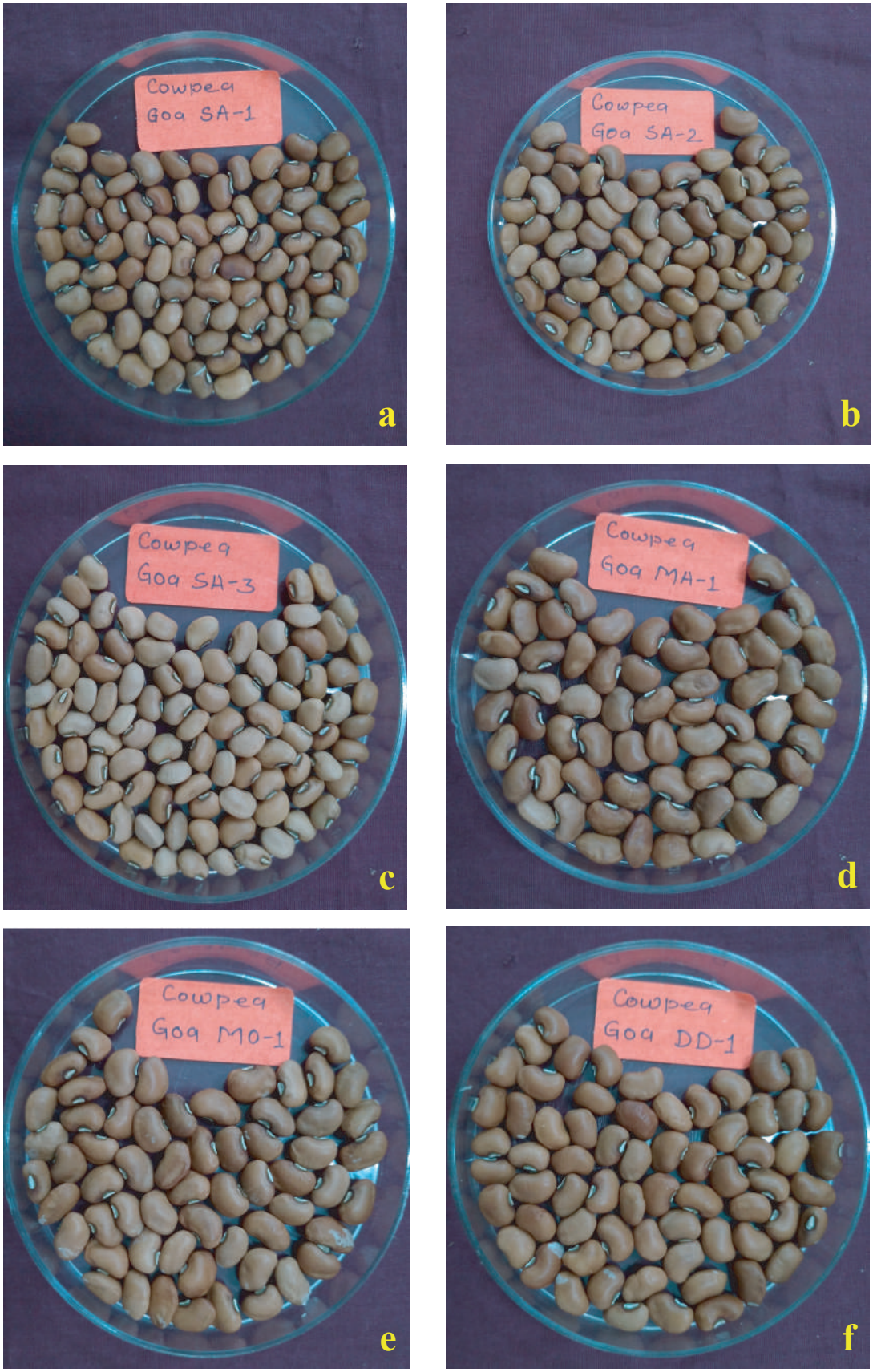


Plate 3.8: *Vigna unguiculata* cultivars collected from agricultural fields of Goa. **a:** Sangolda-I= Goa SA-1, **b:** Sangolda-II= Goa SA-2, **c:** Sangolda-III= Goa SA-3, **d:** Macasana= Goa MA-1, **e:** Moira= Goa MO-1, **f:** Dadachiwadi Dhargalim= Goa.

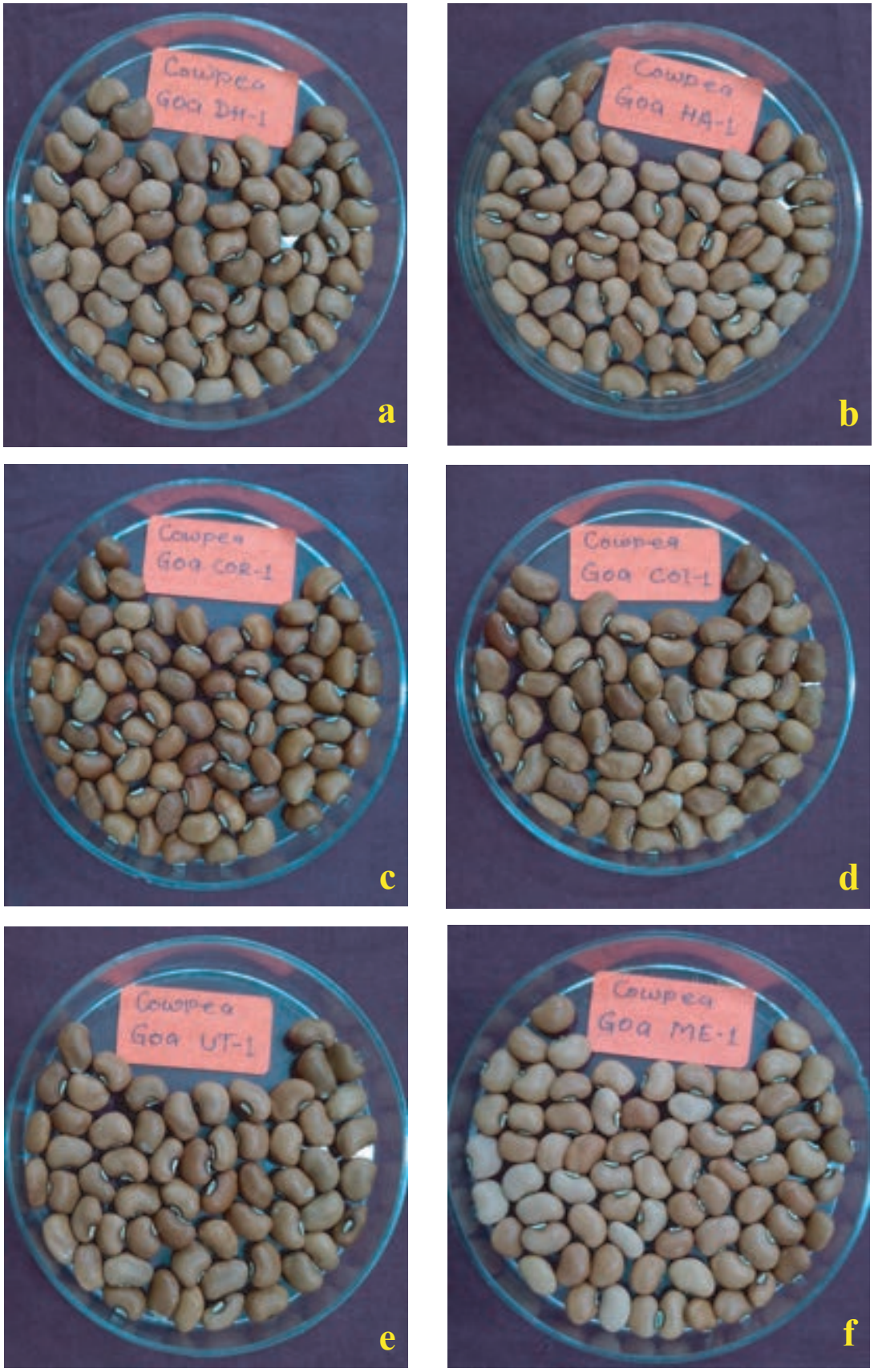


Plate 3.9: *Vigna unguiculata* cultivars collected from agricultural fields of Goa. **a:** Deulwada Harmal= Goa DH-1, **b:** Harmal Pernem= Goa HA-1, **c:** Corjuem= Goa COR-1, **d:** Coimavaddo Aldona= Goa COI-1, **e:** Utorda= Goa UT-1, **f:** Mencurem= Goa ME-1.

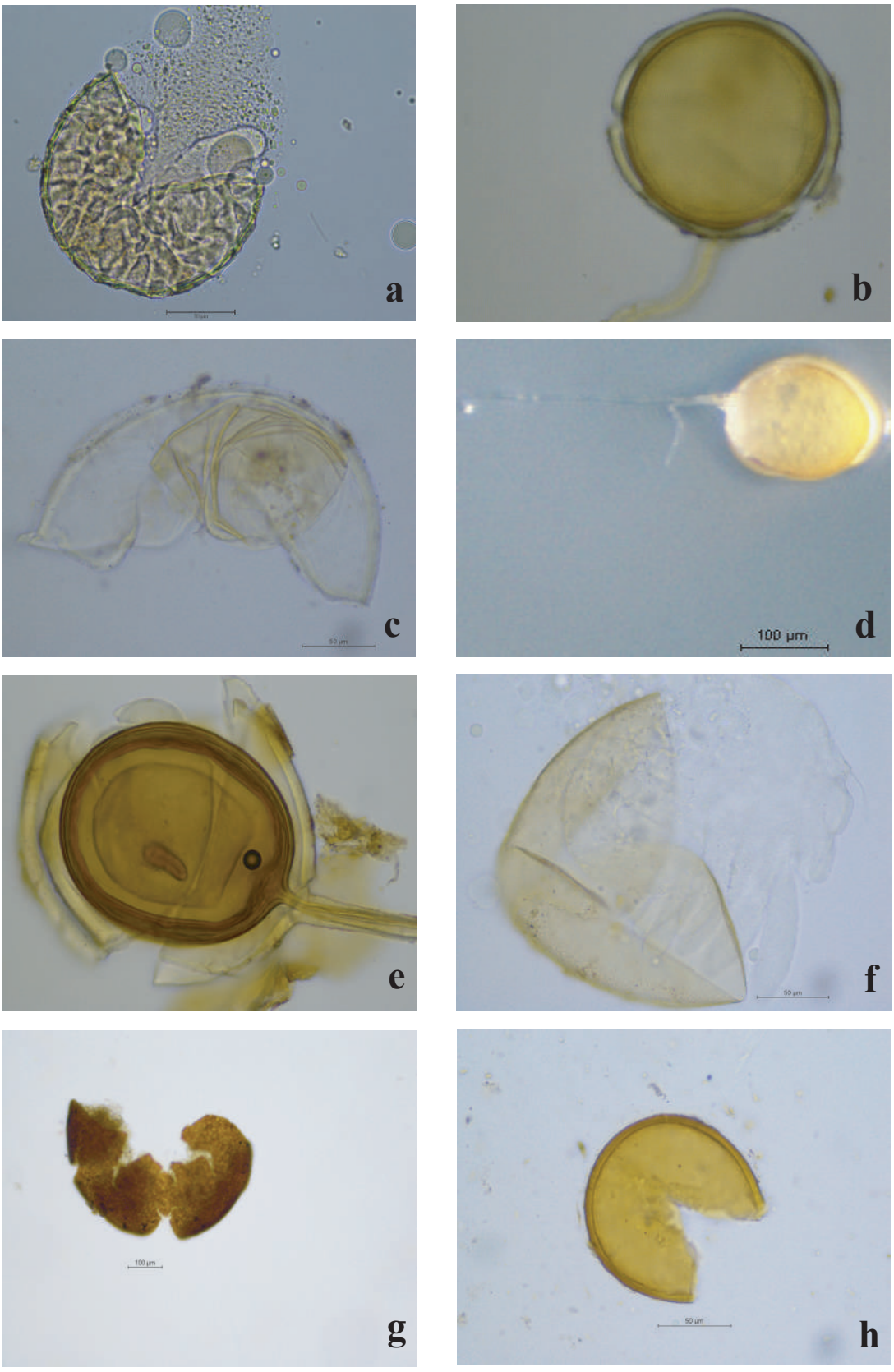


Plate 5.2: Micrographs of AM fungal species isolated from selected study sites. **a:** *Acaulospora myriocarpa*, **b:** *Glomus microaggregatum*, **c:** *Acaulospora delicata*, **d:** *Funneliformis mosseae*, **e:** *Glomus fasciculatum*, **f:** *Acaulospora mellea*, **g:** *A. foveata*, **h:** *A. polonica* (Scale bar: **i-k = 50 µm; l= 100 µm; m-p = 50 µm**).

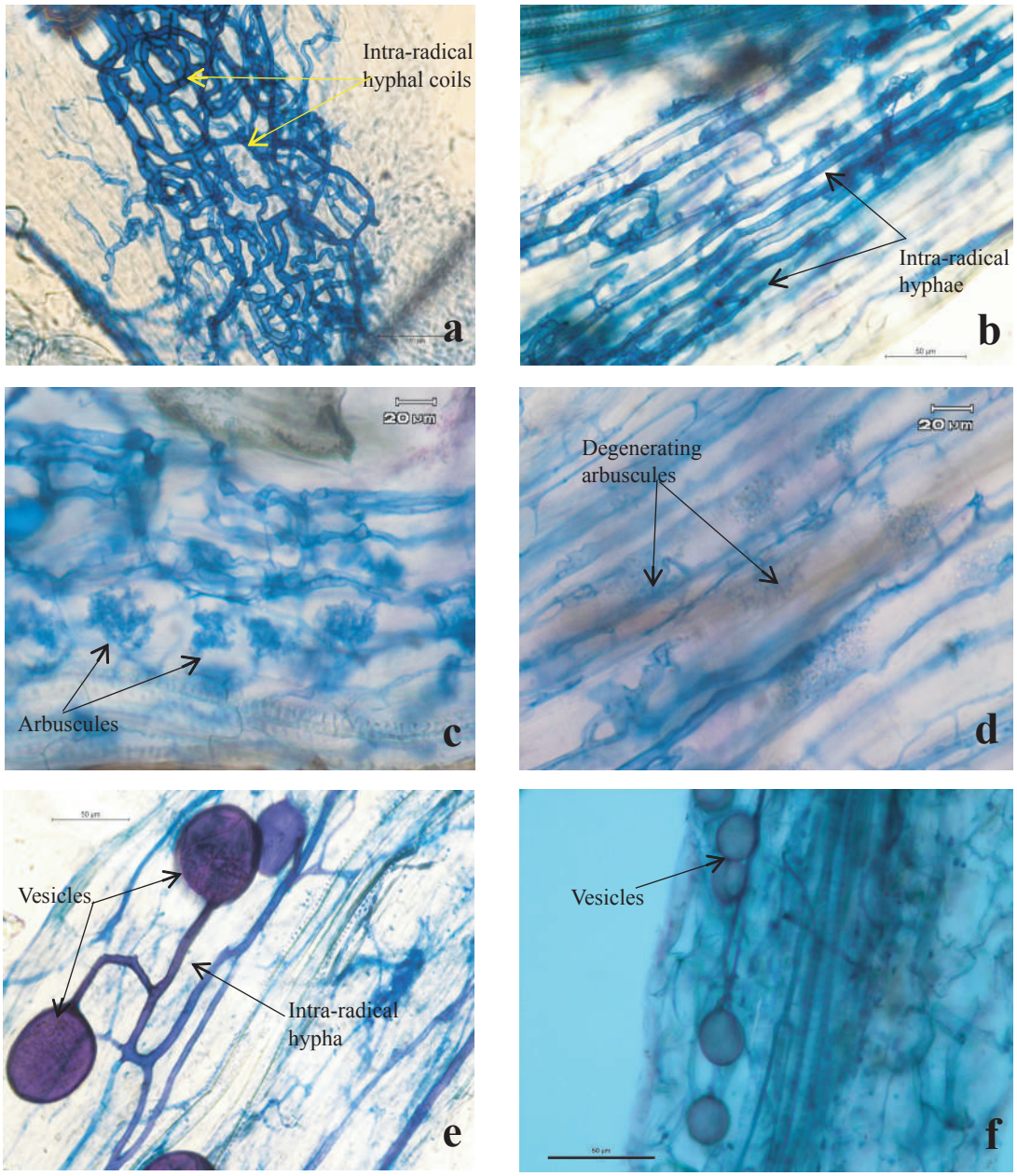


Plate 5.3: Micrographs of root colonization in AM fungal species from selected study sites. **a:** Intra-radical hyphal coils in roots of *V. unguiculata* (L.) Walp, **b:** Intra-radical hyphae, **c:** Arbuscules, **d:** Degenerating arbuscules, **e:** Vesicles, **f:** Vesicles.

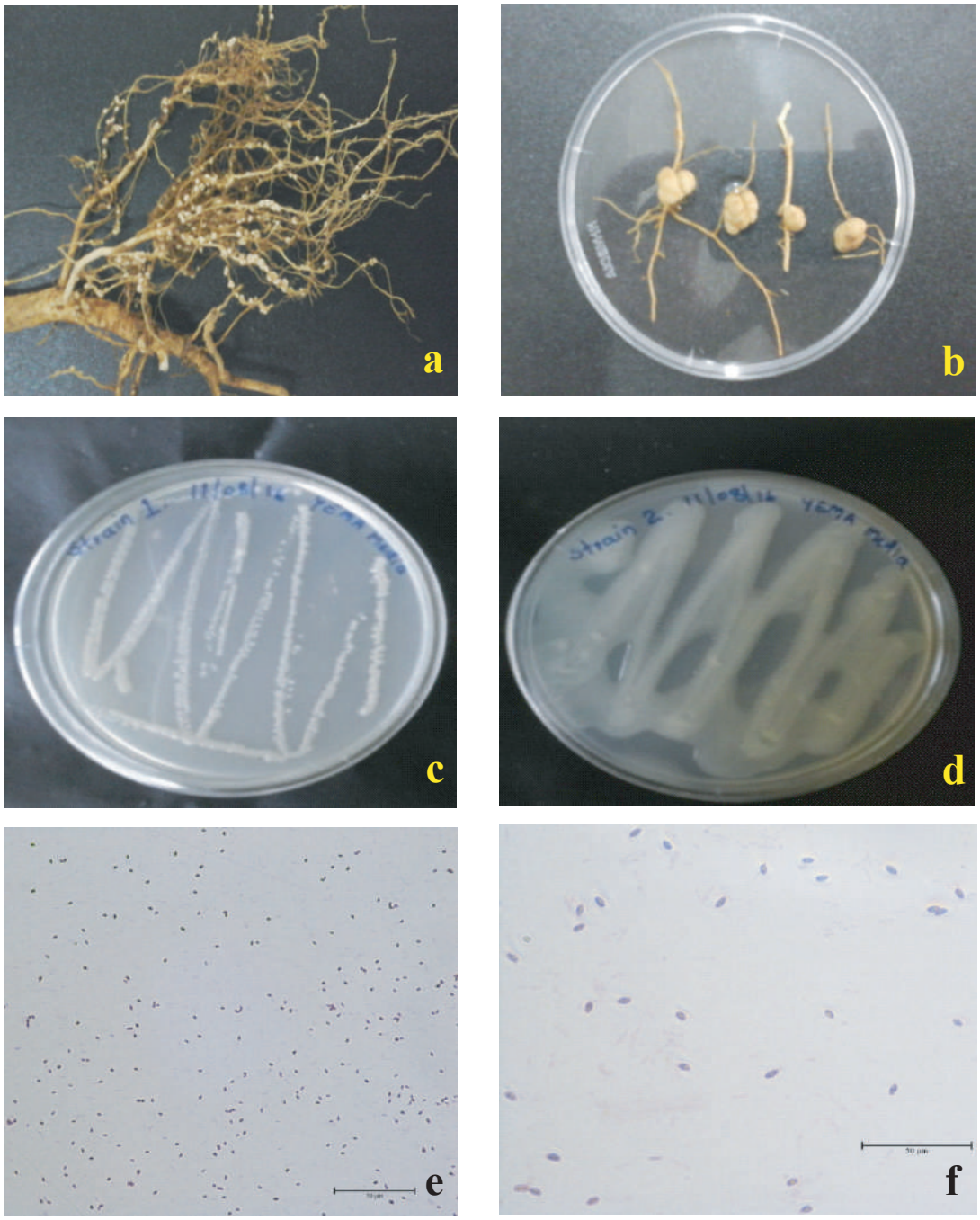


Plate 6.1: Rhizobial strains isolated from root nodules of *V. unguiculata*. **a:** Cowpea root nodules, **b:** Isolated nodules, **c:** GUBRS1, **d:** GUBRS2, **e-f:** Gram stained bacteria.

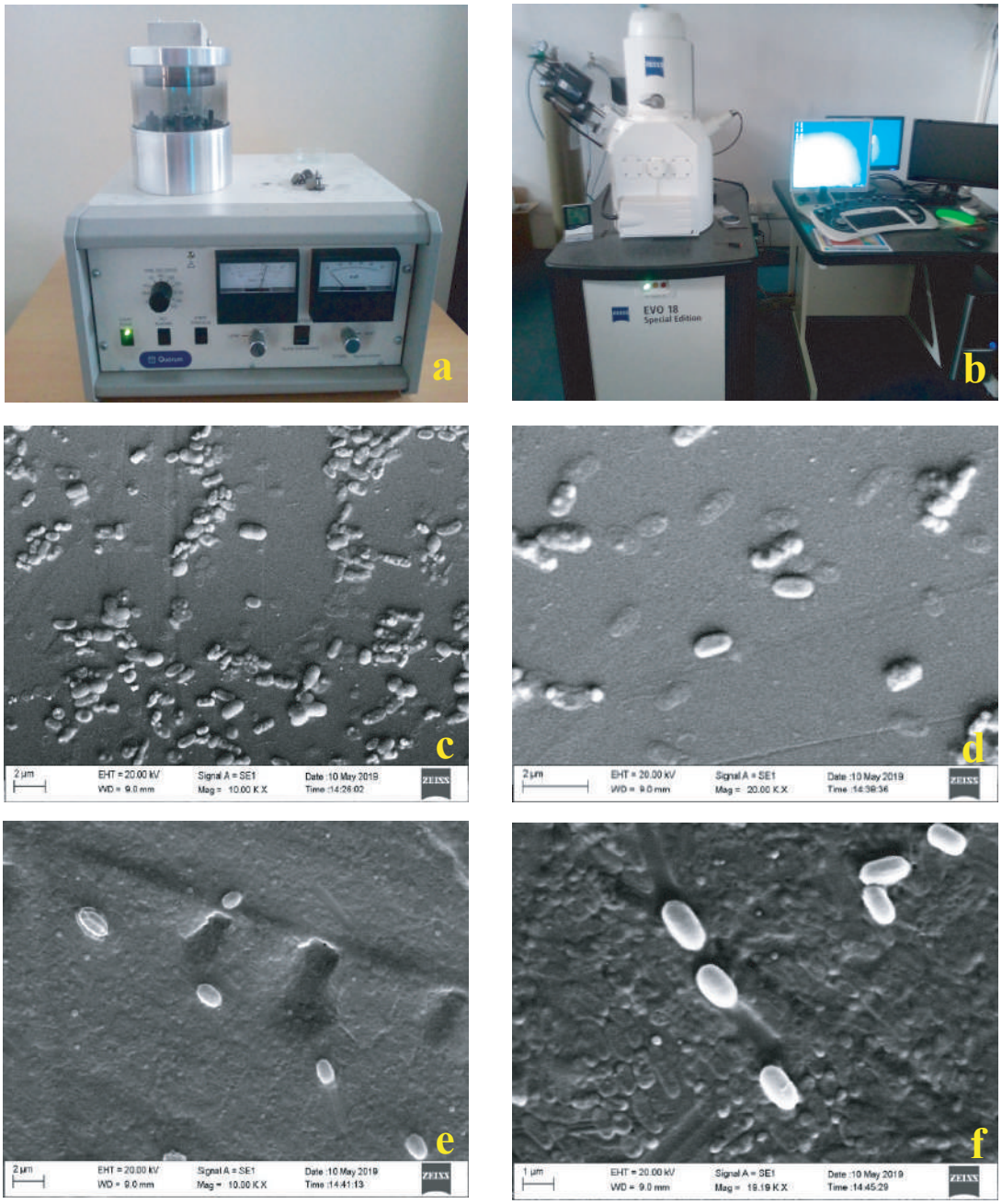


Plate 6.2: Microscopic identification of the *Bradyrhizobium* species using Scanning Electron Microscope (SEM). **a:** Gold-Palladium Sputter coater, **b:** SEM instrument Carl Zeiss EVO 18, **c-f:** SEM images of *Bradyrhizobium* species, **c-d:** GUBRS1, **e-f:** GUBRS2.

Plate 6.3: Nodulation test using the identified isolates. **a:** *Bradyrhizobium* spp. broth culture, **b:** Sterilized *V. unguiculata* seeds, **c:** Sterilized cowpea seedlings grown in sterile sand along with the two isolated GUBRS1 and GUBRS2 strains, **d:** Cowpea roots with root nodules.

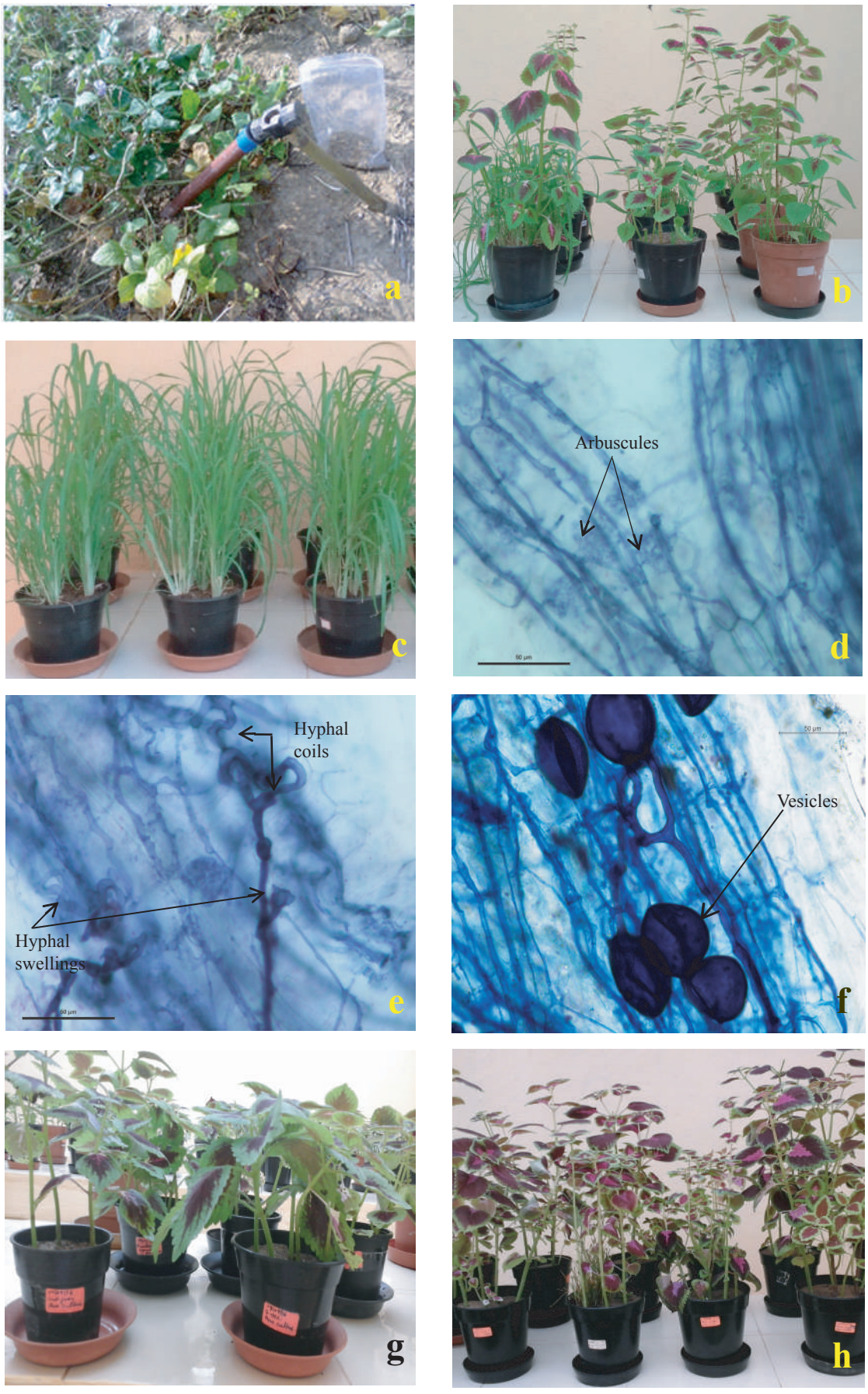


Plate 7.1: Preparation of pure culture inoculum using trap and monospecific cultures. **a:** Rhizosphere soil sample, **b-c:** Trap cultures, **d-f:** Roots colonization in trap cultures, **g-h:** Monospecific cultures.

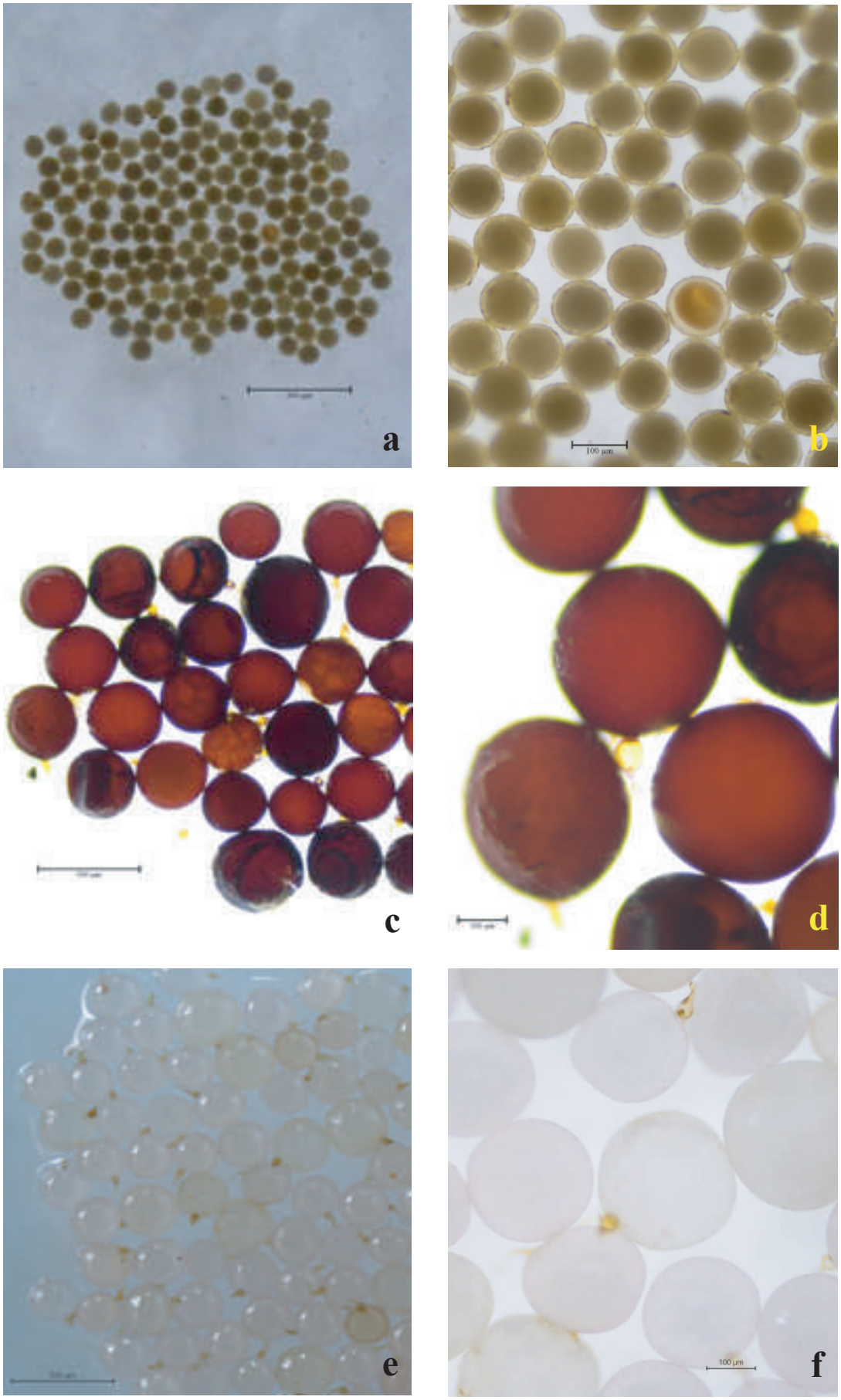


Plate 7.2: Spores isolated from monospecific cultures. **a-b:** *Acaulospora myriocarpa,* **c-d:** *Racocetra gregaria,* **e-f:** *Gigaspora albida* (Scale bar: **a, c, & e=500 µm; b, d, & f= 100 µm**).

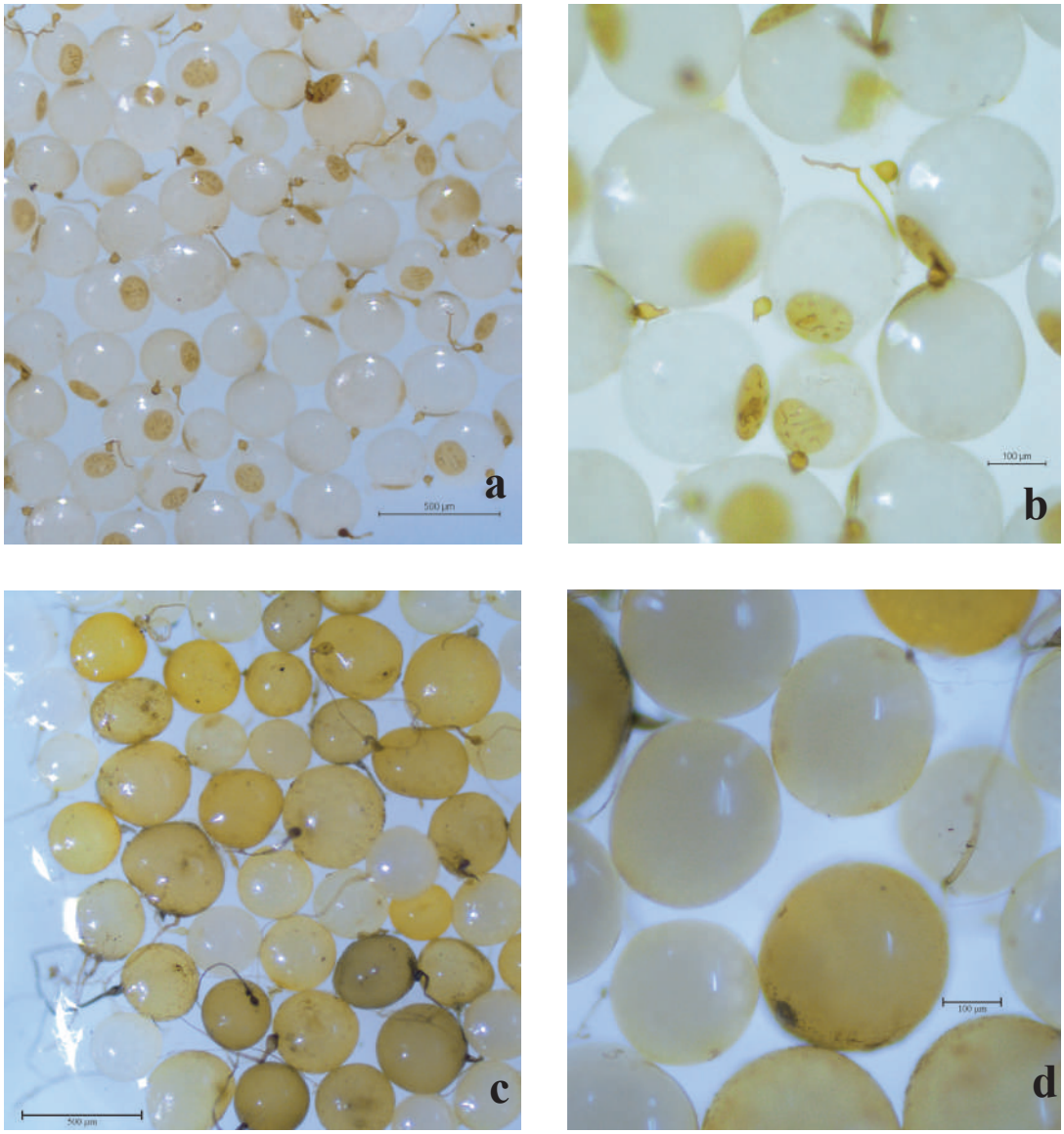


Plate 7.3: Spores isolated from monospecific cultures. **a-b:** *Scutellospora scutata,* **c-d:** *Gigaspora decipiens* (Scale bar: **a & c= 500 µm; b & d= 100 µm**)

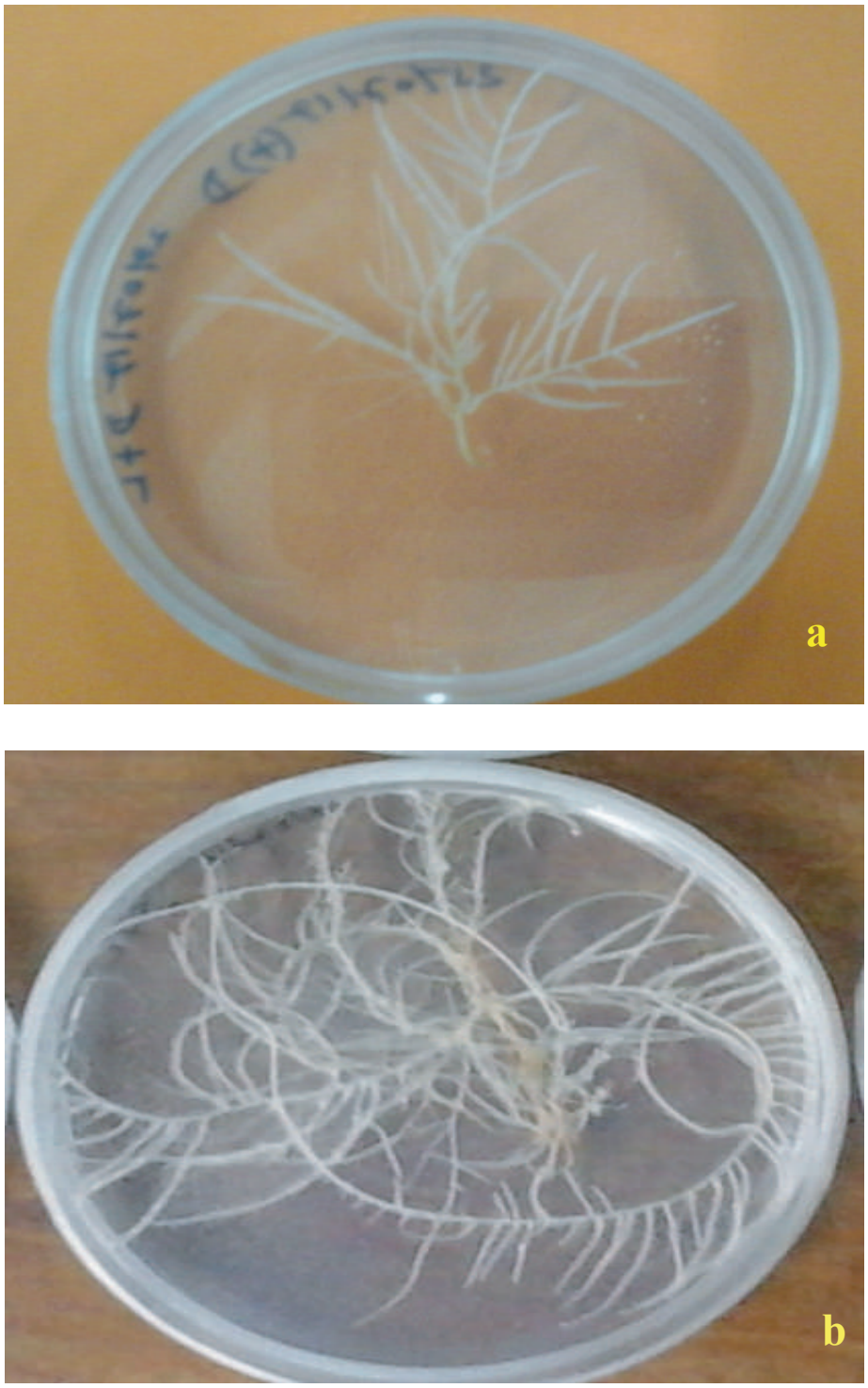


Plate 7.4: Ri T-DNA transformed roots growing on MSR media. **a:** *Linum usitatissimum* (L.) *Linum* Ri T-DNA transformed roots, **b:** *Cichorium intybus* (L.) Chicory Ri T-DNA transformed roots

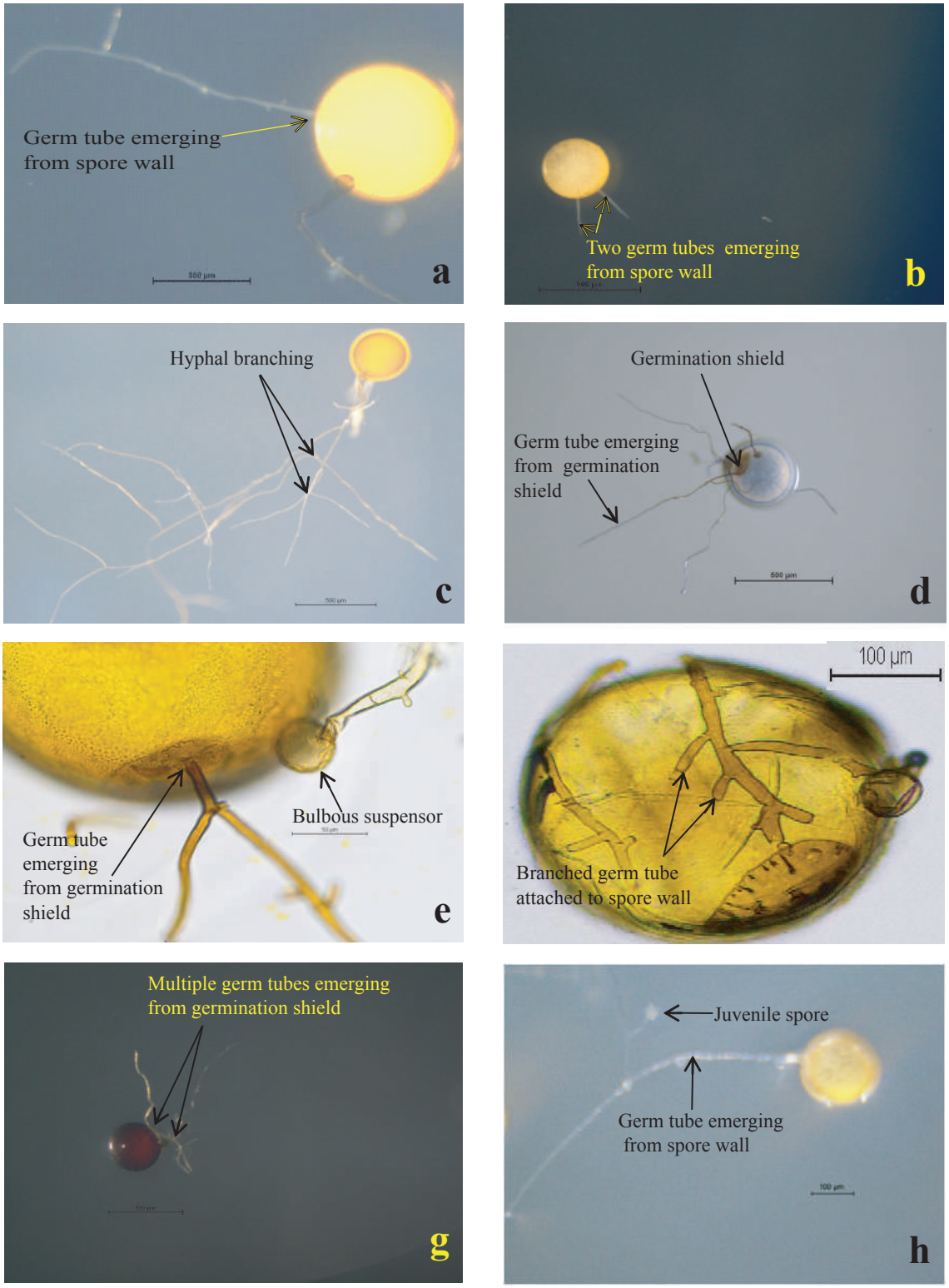


Plate 7.5: *In vitro* germination of AM fungal propagules on MSR (-sucrose) media. **a-c:** *Gigaspora decipiens,* **d-f:** *Scutellospora scutata,* **g:** *Racocetra gregaria,* **h:** *Rhizoglomus intraradices.*

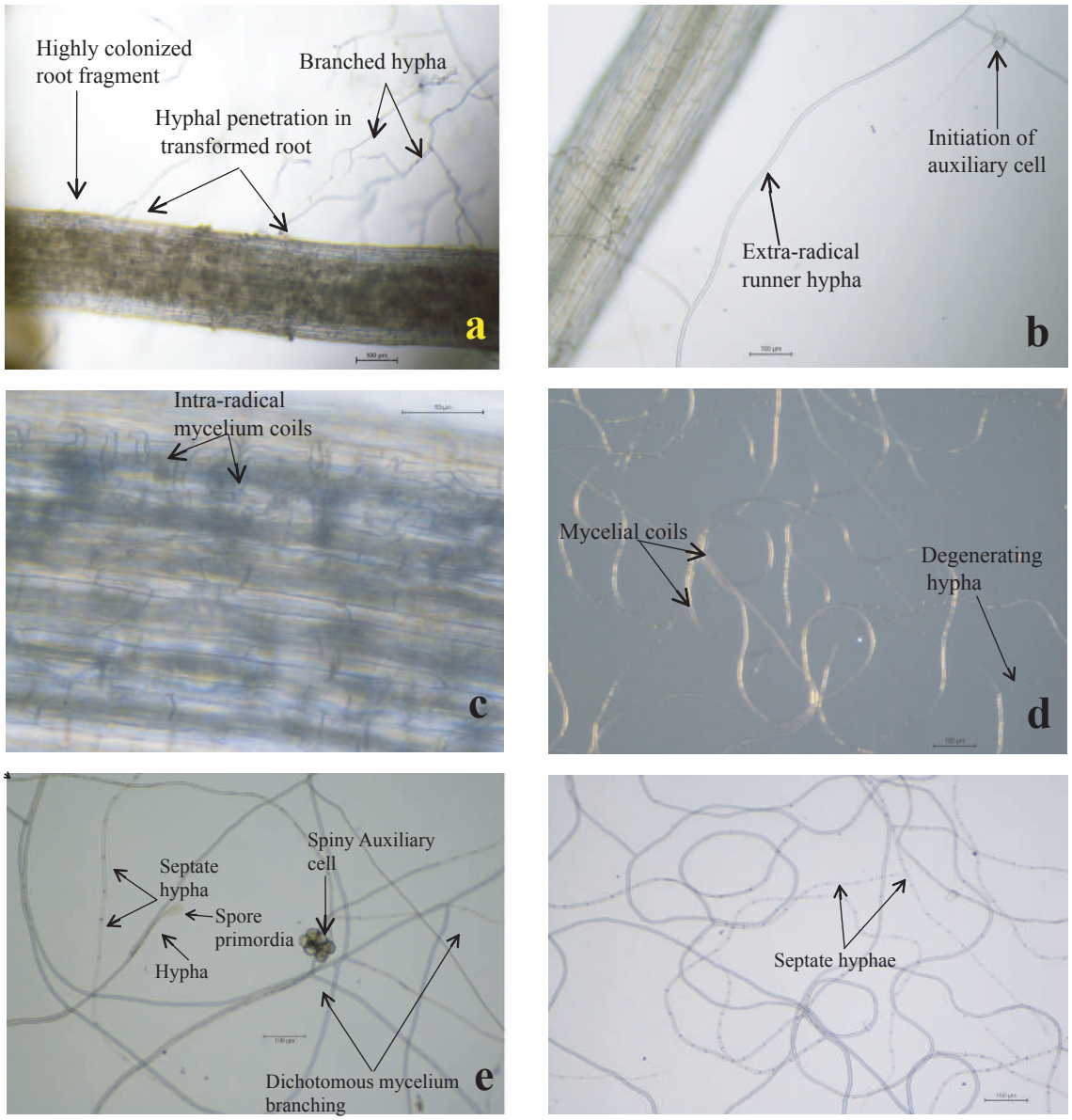


Plate 7.6: Monoxenic culture of *Gigaspora decipiens* with Ri T-DNA roots of *Linum* on MSR media. **a:** Highly colonized root, **b:** Initiation of auxiliary cells, **c:** Intra-radical coil formation, **d:** Hyphal coils and degenerating hyphae, **e:** Dichotomous branching, auxiliary cells and spore primordia, **f:** Hyphal septation

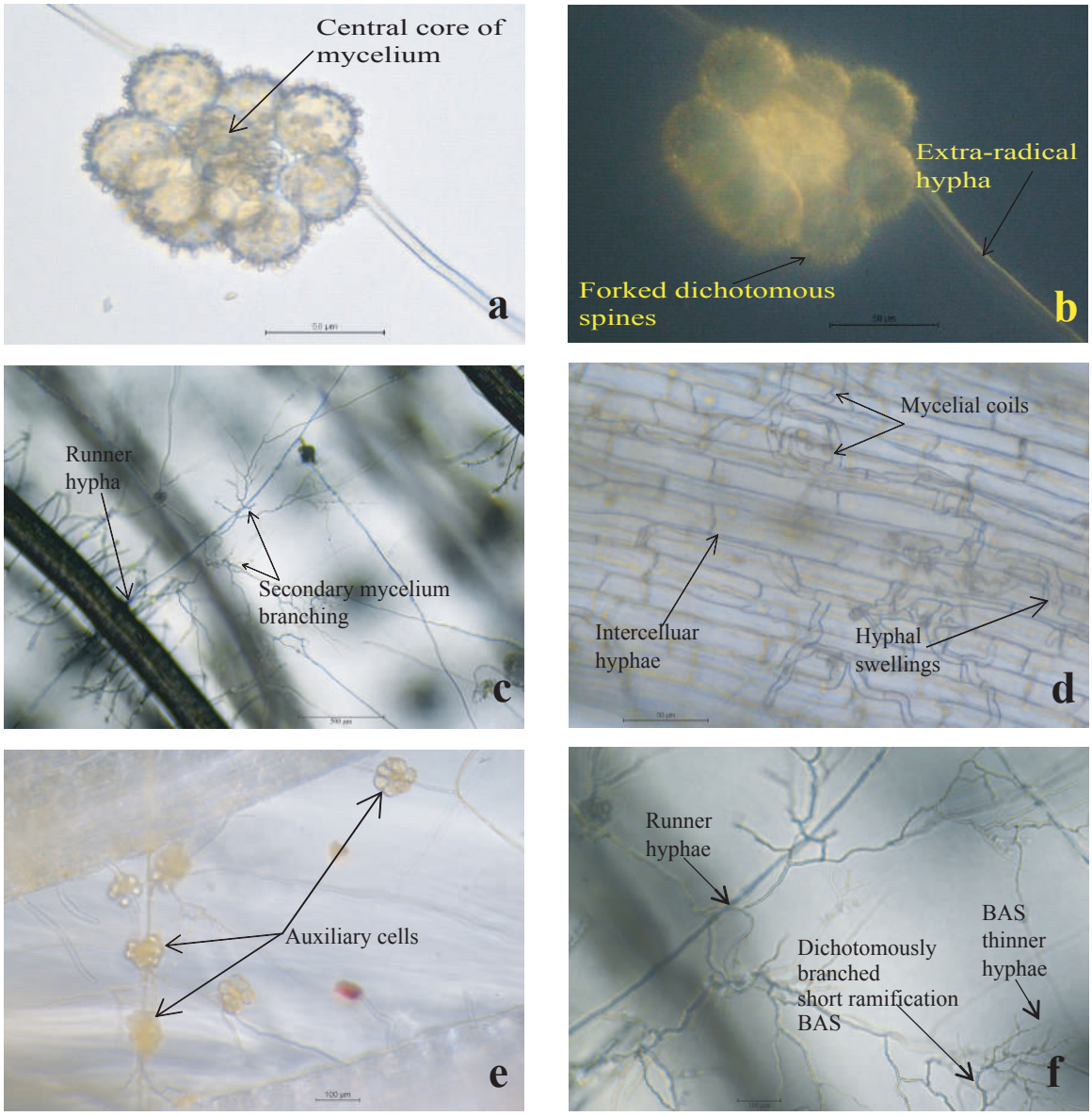


Plate 7.7: Monoxenic culture of *Gigaspora decipiens* with Ri T-DNA roots of *Linum* on MSR media. **a-b:** Auxiliary cells, **c:** Colonized root, **d:** Hyphal swellings, inter- and intra-cellular hyphae, **e:** Auxiliary cells formation, **f:** Runner hyphae and BAS.

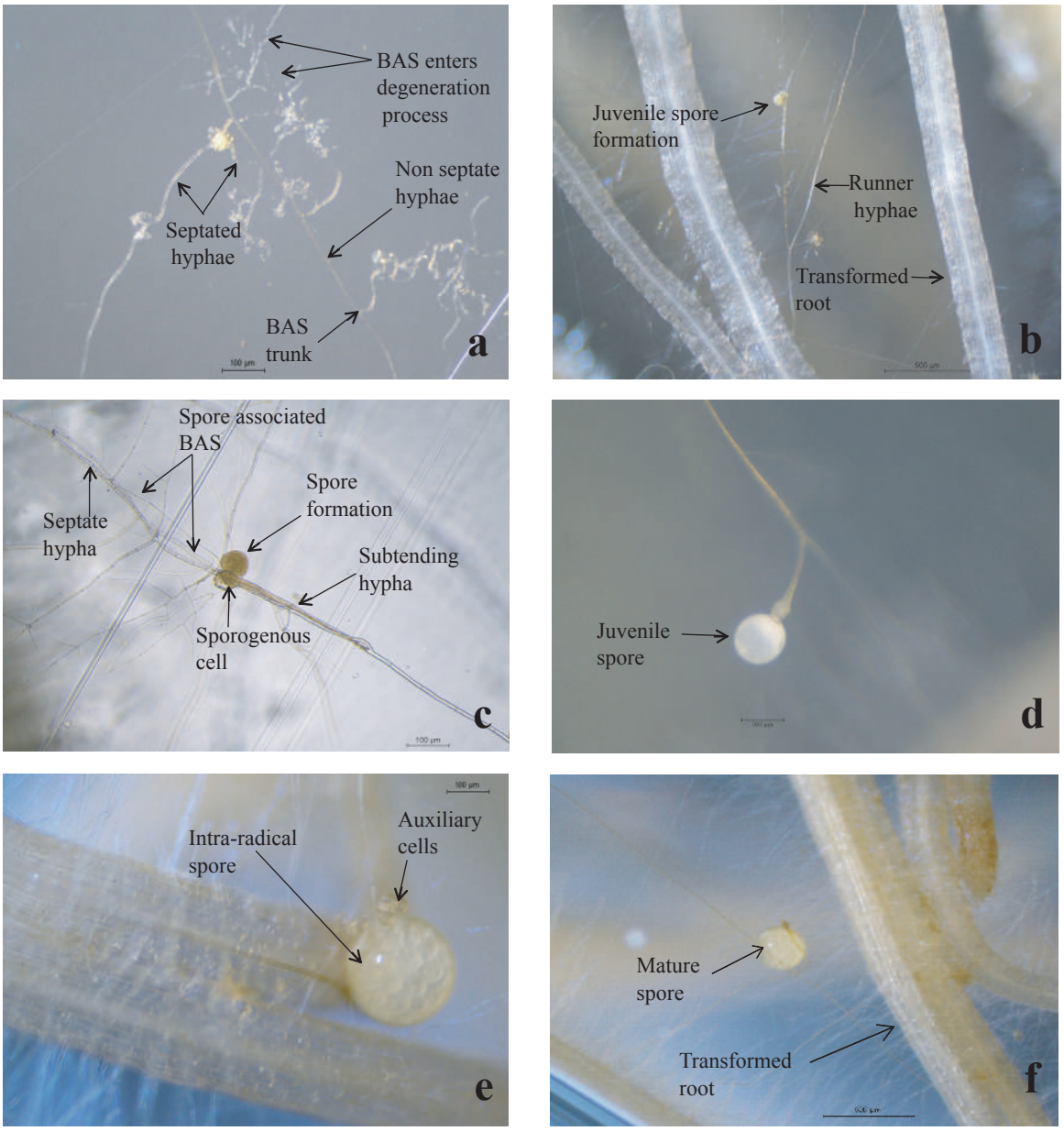


Plate 7.8: Monoxenic culture of *Gigaspora decipiens* with Ri T-DNA roots of *Linum* on MSR media. **a-f:** Developmental stages formation of spore in *Gi. decipiens*.

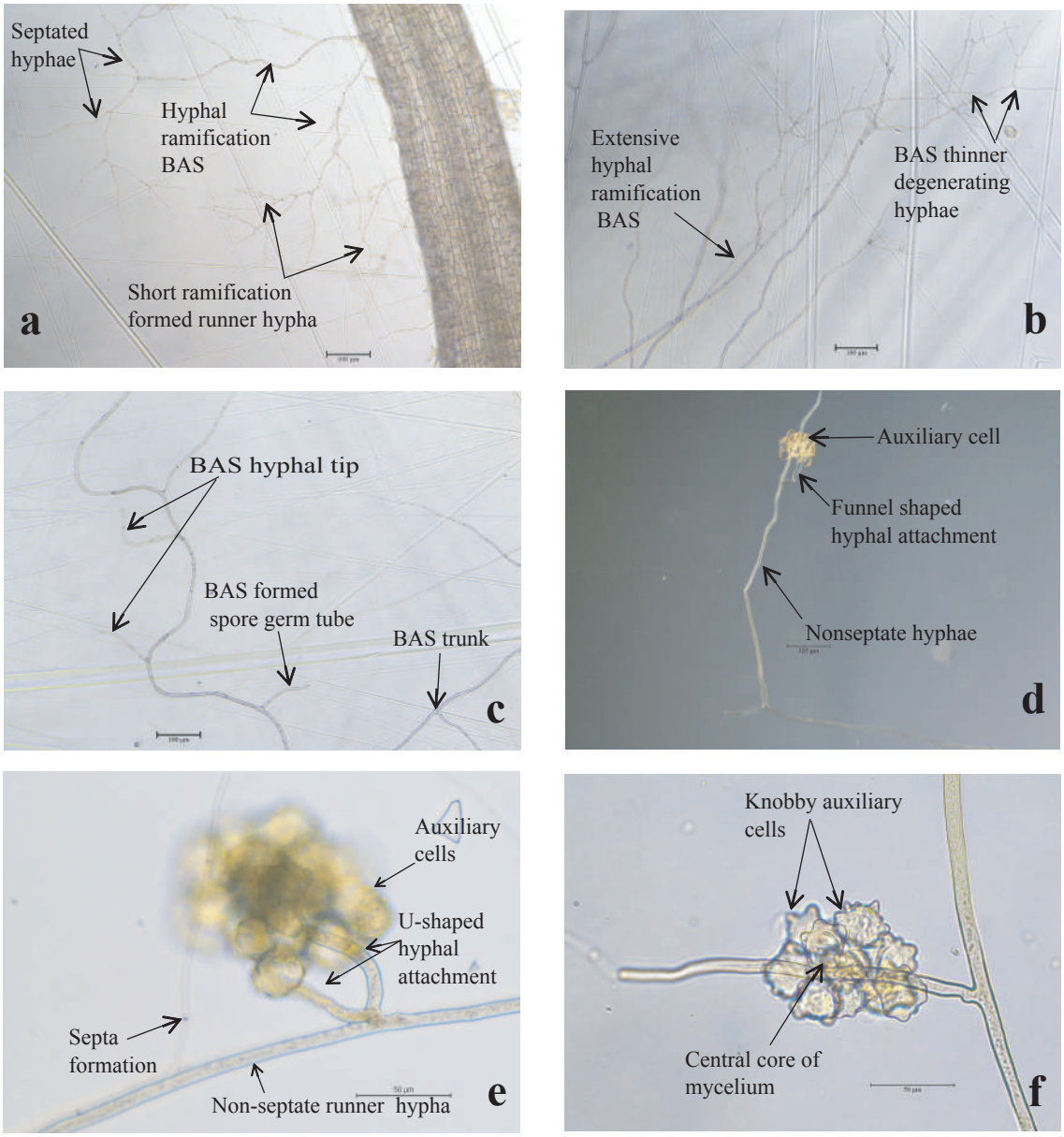


Plate 7.9: Monoxenic culture of *Racocetra gregaria* with Ri T-DNA roots of *Linum* on MSR media. **a:** Colonized transformed root, **b:** Formation of thinner hyphae through dichotomous branching, **c:** Short ramification formed by a spore germ tube, **d-f:** Knobby auxiliary cells produced on coiled hyphae.

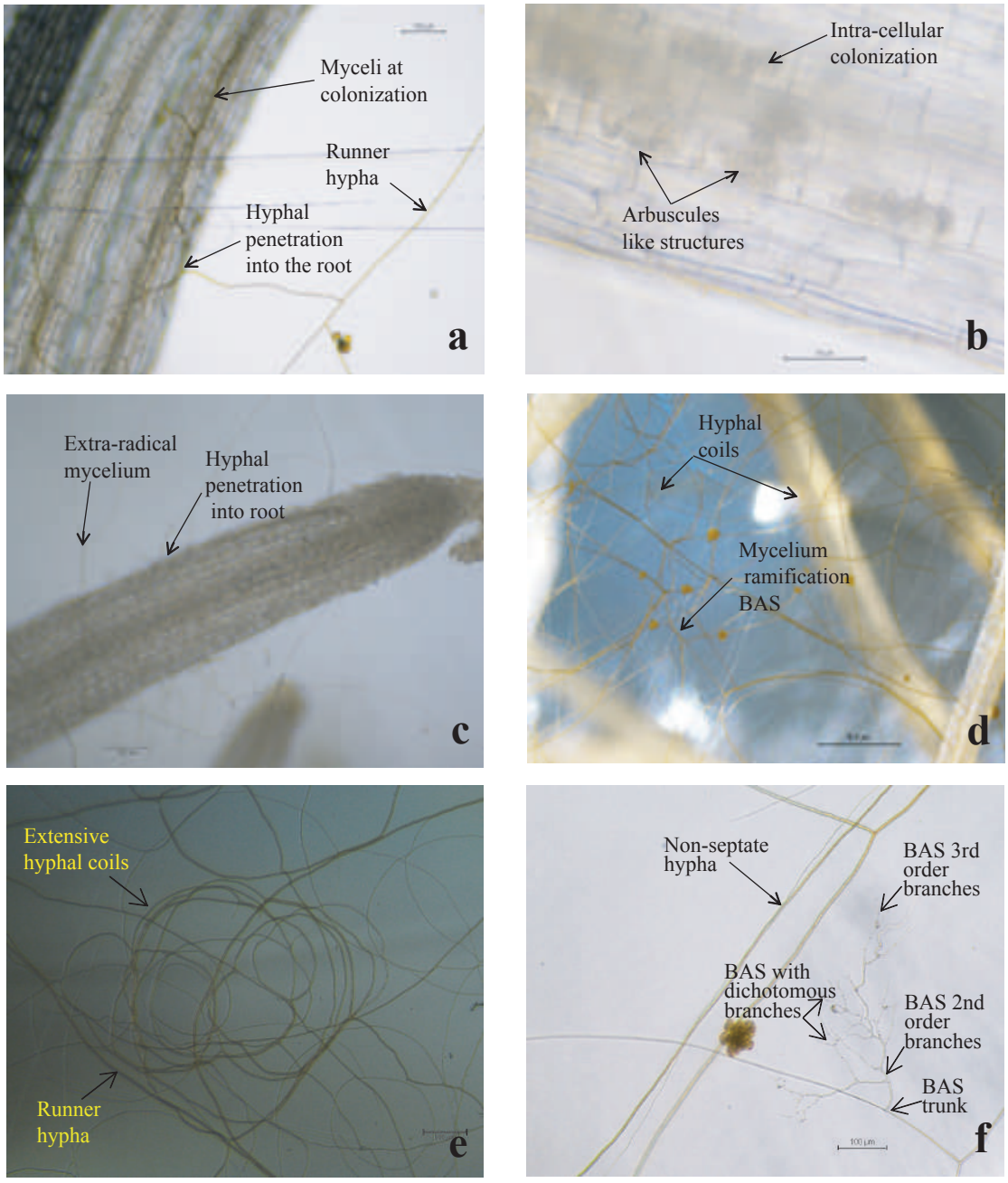


Plate 7.10: Monoxenic culture of *Scutellospora scutata* with Ri T-DNA roots of *Linum* on MSR media. **a:** Colonization in transformed root, **b:** Arbuscule like structures in colonized root, **c:** Extra-radical mycelium in colonized root, **d-e:** Extensive hyphal coils, **f:** Morphogenesis of a branched absorbing structure (BAS).

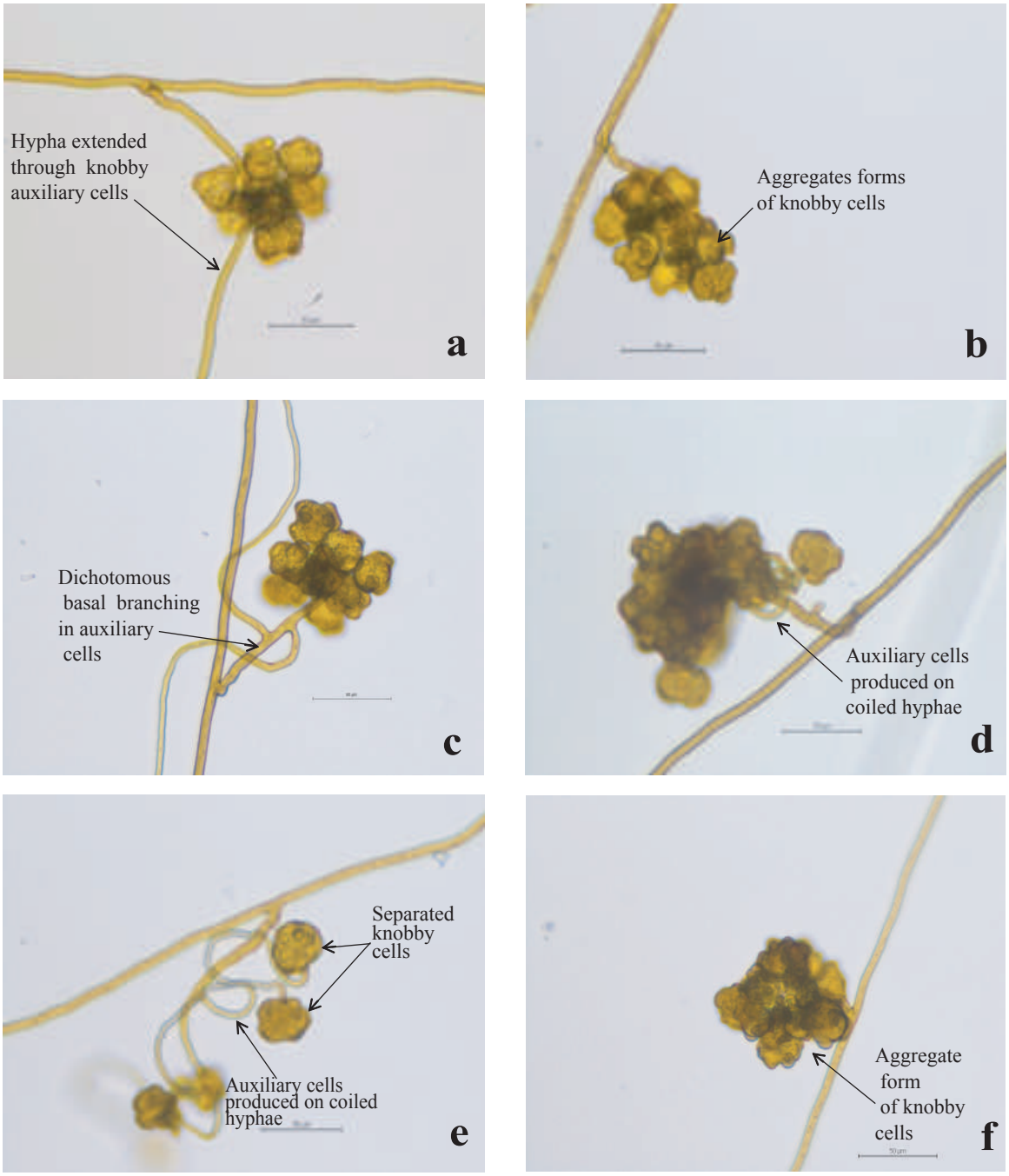


Plate 7.11: Monoxenic culture of *Scutellospora scutata* with Ri T-DNA roots of *Linum* on MSR media. **a-f:** Development of highly variable knobby auxiliary cells on transformed colonized roots.

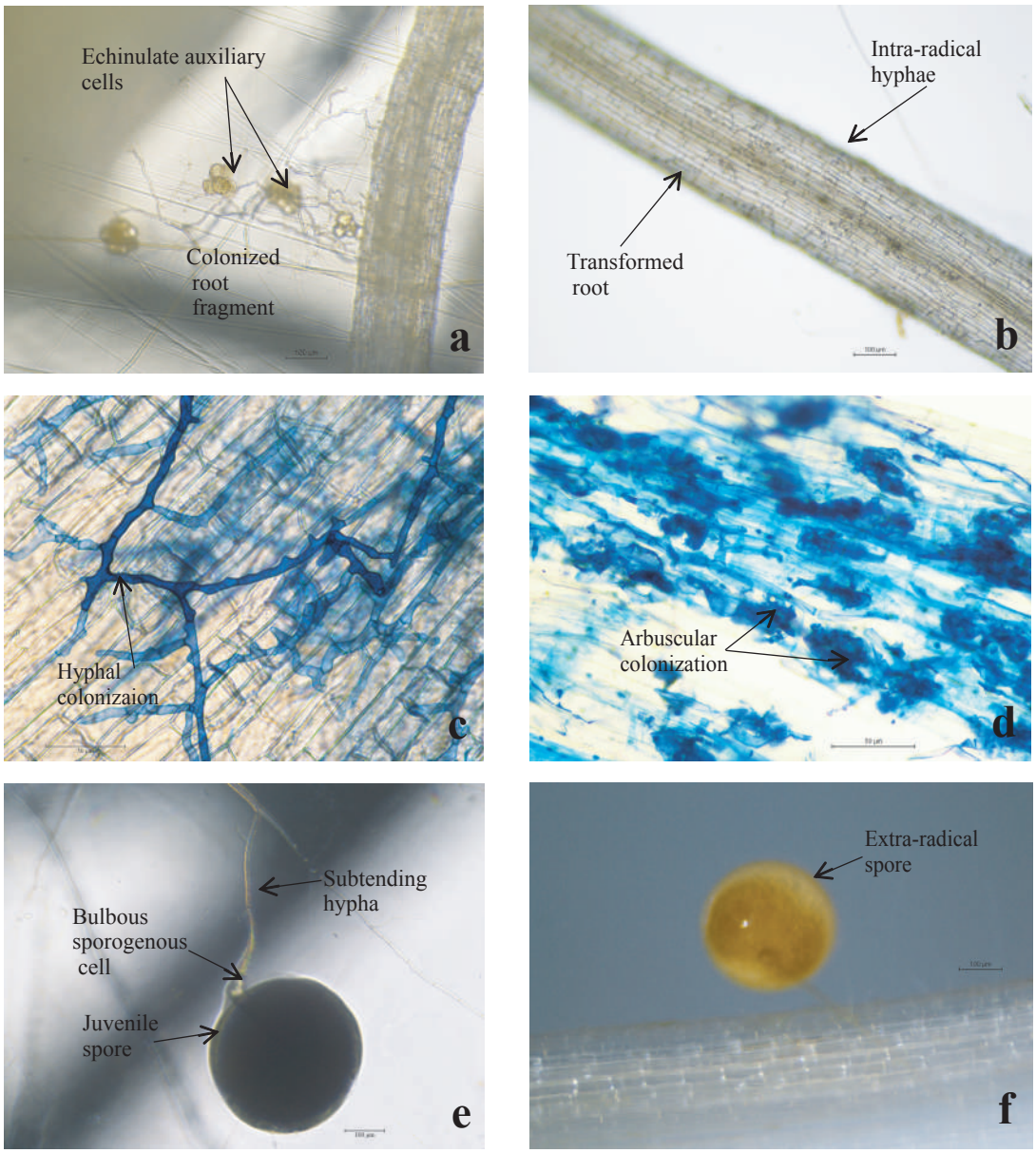


Plate 8.1: Assessment of monoxenic germination potential. **a-b:** Colonized root fragments, **c:** Hyphal colonization, **d:** Arbuscular colonization, **e:** *In vitro* sporulation in *Gigaspora decipiens,* **f:** Extra-radical spore.

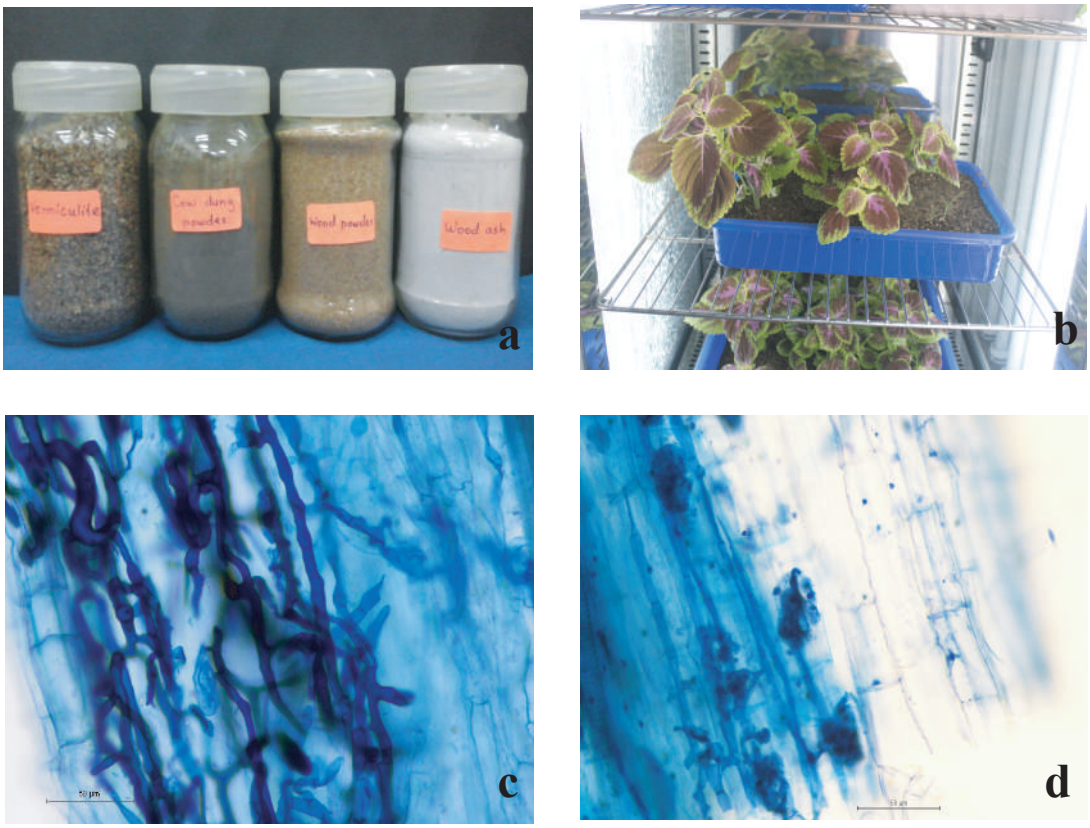


Plate 8.2: Assessment of infectivity potential of *in vitro* prepared inocula in carrier formulation. **a:** Carrier materials, **b:** Carrier based mass production in Bio-incubator chamber, **c-d:** AM fungal colonization.

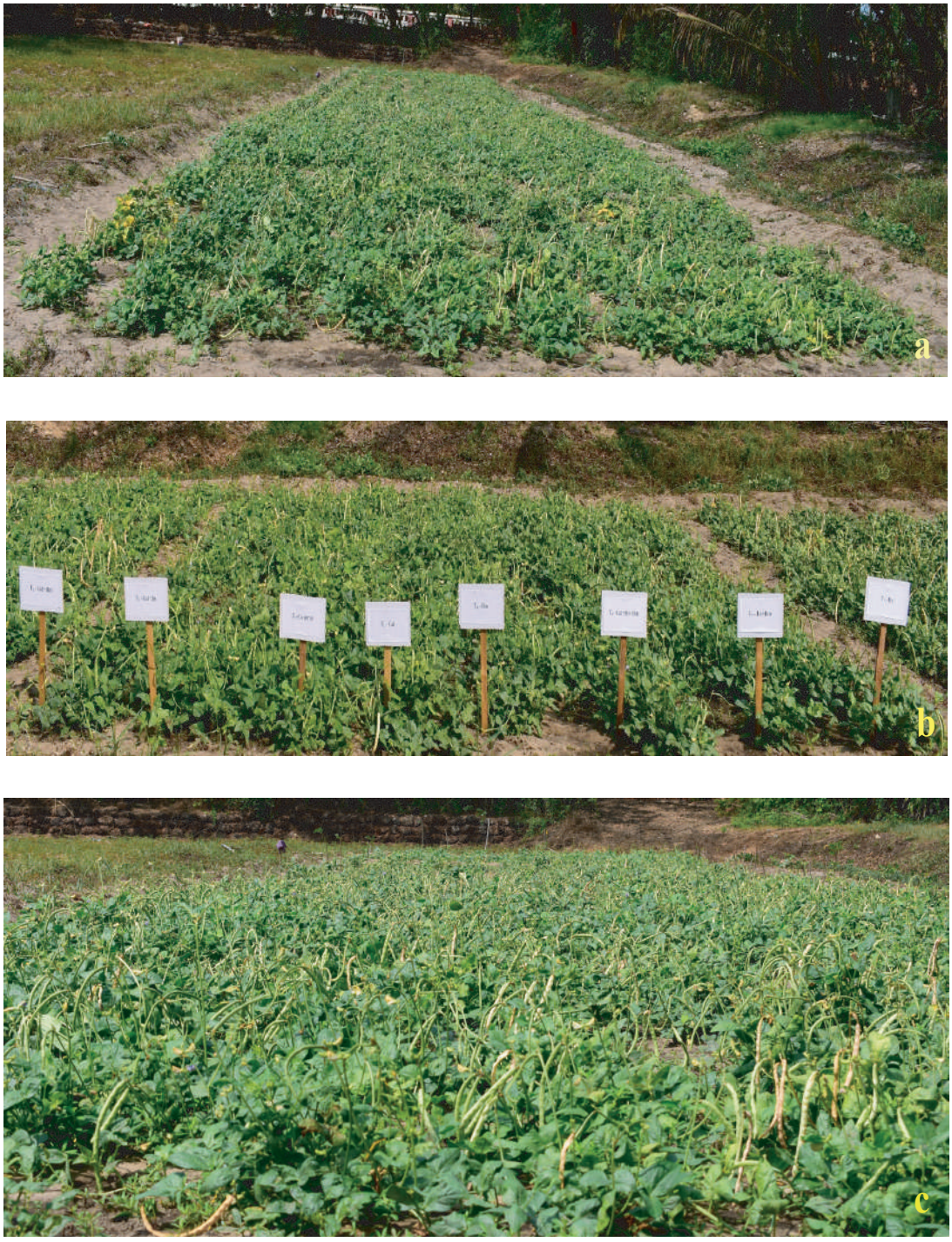


Plate 9.3: Overview of field bio-inoculants experimental study. **a-b:** Cowpea grown plot employed with replicated randomized block design, **c:** Closer view of cowpea plants with pods.

Plate 9.4: Field grown cowpea amended with bio-inoculants. **a: T1:** Control, **b: T2:** *Gi. decipiens*, **c: T3:** *Bradyrhizobium* sp., **d: T4:** *Bacillus methylotrophicus* (Goa Bio-1 culture).

Plate 9.5: Field grown cowpea amended with bio-inoculants. **a: T5:** *Gi. decipiens* + *Bradyrhizobium* sp., **b: T6:** *Gi. decipiens* + *B. methylotrophicus*, **c: T7:** *Bradyrhizobium* sp. + *B. methylotrophicus*, **d: T8:** *Gi. decipiens* + *Bradyrhizobium* sp. + *B. methylotrophicus.*

KAVAKA 53:.....-....(2019)

In vitro cultivation of *Gigaspora decipiens* using transformed roots of *Linum usitatissimum*

Dhillan M. Velip and B.F. Rodrigues*
Department of Botany, Goa University, Goa 403 206
**Corresponding author Email:felinov@gmail.com*
(Submitted on November 12, 2019; Accepted on December 17, 2019)

ABSTRACT

Symbiosis between arbuscular mycorrhizal fungi (AMF) and higher plants provides a wide scope for its use as biofertilizer. Mass multiplication of pure AMF cultures however, has always been a challenge. Use of transformed roots for the establishment of monoxenic cultures of AMF is being done in recent years but with a low success rate with regard to spore production *in vitro*. The present study exhibits a successful attempt towards *in vitro* culturing and sporulation of *Gigaspora decipiens* Hall & Abbott in transformed roots of *Linum usitatissimum* L. (Flax) Also, the present study describes a technique wherein spore germination and *in vitro* root colonization can be brought about in the same Petri plate rather than transferring a prior germinated AM spore among the t-DNA roots. This technique minimizes the effect of relocation of germinating spores thereby hastening root colonization.

Keywords: Sporulation, culturing, AMF, monoxenic culture, transformed roots,

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are one of the most abundant groups of symbiotic organisms. They are involved in supplying nutrients to about 80% of terrestrial plants thereby improving their growth and vigour (Smith and Read, 2008). This feature projects AMF as high potential candidates for use as biofertilizers.

The glitch in use of AMF as biofertilizers lies in the fact that they are obligatory biotrophs. They cannot be grown or multiplied on artificial medium. Researchers have found a solution to this difficulty through development of monoxenic cultures using *in vitro* grown transformed roots as a symbiotic partner for AMF (Bécard and Fortin, 1988; Mosse and Hepper, 1975; Fortin *et al*., 2002). Monoxenic cultures of AMF so developed ascertains purity of the cultures and enables continuous monitoring of the colonies to observe mycelial development and spore initiation and maturation (Tahir, 2003; Pawlowska *et al*., 1999; Akimaya *et al.,* 2005; Calvet *et al*., 2013; Voets *et al*., 2009). More importantly it helps in molecular, physiological and taxonomic studies of the AMF species (Croll *et al*., 2008; Kokkoris and Hart, 2019; Luginbuehl *et al*., 2017).

This technique is however, in its infancy with not many species of AMF in culture. Also few of the cultures have difficulty in sporulating *in vitro* (Karandashov *et al*., 2000). The few strains that have been successfully brought into monoxenic cultures mainly belong to the genera *Glomus* and *Gigaspora*. One or more strains of *Scutellospora* and *Acaulospora* have also been cultured *in vitro* (Dalpé and Declerck, 2002; de Souza and Declerck, 2003). Researchers have identified humidity, light, CO_2, temperature and pH as some of the important physical factors while substrate composition and nutrient availability as the nutritional factors involved in successful establishment of monoxenic cultures (Bécard *et al*., 1992; Maia and Yano-Melo, 2001). Besides, factors such as presence of root exudates or contaminants have also been found to significantly affect growth and sporulation of AMF in monoxenic cultures.

The present study was aimed to establish a monoxenic culture of *Gigaspora decipiens* in transformed roots of *Linum usitatissimum* L. and to induce sporulation in them (**Fig. 1: a-l**).

MATERIAL AND METHODS

Source of AMF: Rhizosphere soil associated with *Vigna unguiculata* (L.) Walp. collected from agriculture fields was selected as source of AMF. Spores of AMF were isolated using the wet sieving and decanting technique (Gerdemann and Nicolson, 1963). Among the isolated species, *Gi. decipiens* was dominant.

Further, the spores of *Gi. decipiens* were multiplied through trap culture in Coleus plant [*Plectranthus scutellarioides* (L.) R.] in the polyhouse (Goa University Arbuscular Mycorrhizal Culture Collection (GUAMCC)). The plants were grown in pots containing sterilized sand and maintained under controlled polyhouse conditions (25°C, RH 80-90%) for 45 days.

Development of monoxenic cultures: *Gi. decipiens* spores extracted from the trap culture were carefully picked using a stereomicroscope. Under aseptic conditions, the isolated spores were rinsed twice in sterile distilled water and disinfected in 250µL sodium hypochlorite for 5 minutes. This was followed by triple rinsing with sterile distilled water. The spores were finally rinsed in streptomycin sulfate (0.02% w/v) for 10 minutes (Mosse, 1959; Bécard and Fortin, 1988).

Modified StrulluRomand (MSR) medium (pH 6.5) was prepared with and without sucrose. Petri plates were poured with MSR medium with sucrose and allowed to solidify in a slanting position. This was overlayed with a thin layer of MSR medium without sucrose such that part of the MSR medium with sucrose still remained exposed on the surface. Surface-sterilized spores were inoculated on these plates for germination. They were placed in the region of the Petri plate which had MSR medium without sucrose. The Petri plates were incubated in an inverted position in dark at 27°C and monitored daily under a stereomicroscope for germination. The T-DNA transformed roots of *L. usitatissimum* were procured from Prof. Stéphane Declerck, Mycothèque de l'Université Catholique de Louvain (MUCL), Belgium.

Once germination was observed, an actively growing

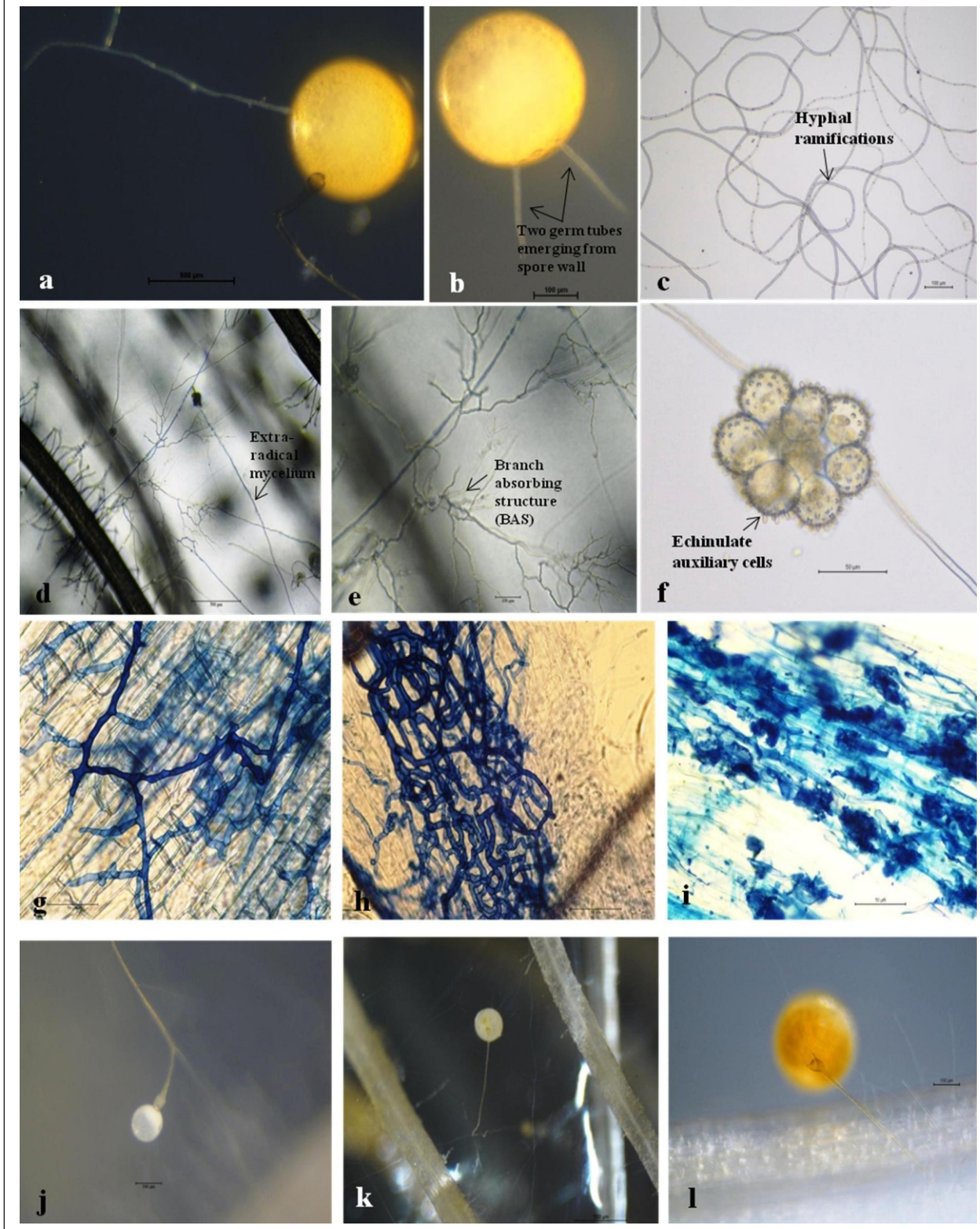


Fig. 1: *In vitro* culture *of Gigaspora decipiens*. **a, b.** spore germination; **c.** hyphal ramifications; **d.** extra-radical mycelium; **e.** branched absorbing structures (BAS); **f.** auxiliary cells; **g, h.** hyphal colonization; **i.** arbuscules; **j-k.** sporulation showing juvenile spores, **l.** mature spore.

transformed root of *L. usitatissimum* with several lateral branches was placed in the vicinity of the germinated spore in the Petri plate which had MSR medium with sucrose. The Petri plates were then again incubated in an inverted position in the dark at 27^0C and monitored after every 4 days under the stereomicroscope for colonization followed by sporulation. After 45-50 days, the roots were stained with trypan blue (Phillips and Hayman, 1970; Giovannetti and Mosse, 1980) to observe root colonization. Spore production of *Gi. decipiens* was evaluated on weekly basis under a stereomicroscope over a period of 6 months.

RESULTS AND DISCUSSION

In vitro spore germination in *Gi. decipiens* was recorded within 3 days of plating on MSR medium which showed emergence of multiple germ tubes (**Fig. 1: a, b**). In an earlier study, Costa *et al*., (2013) reported spore germination in *Gi. decipiens* after 4-5 days.

After 20-25 days of incubation, hyphae grew throughout the Petri plate and developed hyphal ramifications, abundant extra-radical mycelia, branched absorbing structures (BAS) and auxiliary cells (**Fig. 1: c-f**). These various hyphal structures were also observed by earlier workers (Maia and Yano-Melo, 2001; de Souza *et al*., 2005; Costa *et al*., 2013) and were probably formed in response to environmental or nutritional stimulation (Bécard and Fortin, 1988; Bago *et al*., 1998b). It has also been suggested that these structures, particularly the BAS, increase the contact surface of the fungus with the culture medium thereby improving its nutrient absorption rate (Bago *et al*., 1998a). An increased number of BAS by a particular strain suggests its ability of better absorption of the nutrients and thereafter its enhanced delivery to its co-symbiont, the plant and improved plant growth rate. Owing to its high BAS formation, the present strain has a potential to be considered as a candidate for biofertilizer inoculum production.

The germ tubes grew and branched in direction of the transformed roots. The hyphal branching in the culture medium exhibited two patterns of growth, *viz.* apical and lateral. The lateral branches showed the presence of septa. The hyphae successfully colonized the transformed *Linum* roots after 45 days. The hyphal colonization was dense as evident through trypan blue staining (**Fig.1: g, h**). The hyphae penetrated the cells to form arbuscules **Fig.1: i**). Since, both the spore and transformed roots were inoculated on the same Petri plate in regions containing MSR medium without and with sucrose, respectively, the effect of relocation of germinating spores was minimized, thereby speeding root colonization.

Sporulation was observed after 50 to 55 days of growth (**Fig.1: j-l**). On an average 5 spores were observed per Petri plate. This is a significant step in *in vitro* culture of AMF as this step is essential to scale up AMF inoculum production (Declerck *et al*., 2001; Ijdo *et al*., 2011). The ability of *Gi. decipiens* strain under study to sporulate within 2 months of inoculation is yet another highly promising step towards considering the strain for biofertilizer inoculum production.

CONCLUSION

The monoxenic culture of *Gi. decipiens* was successfully established in transformed root of *L. usitatissimum* L. Further the culture produced BAS and spores which are highly desirable characters in considering an AMF strain for biofertilizer inoculum production. This culture is therefore being further developed as inocula for its application as biofertilizer.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge the financial assistance received from the Department of Science, Technology and Environment (DSTE), Government of Goa, to carry out this study.

REFERENCES

Akiyama, K., Matsuzaki, K. and Hayashi, H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature.* **435**: 824-827. doi: 10.1038/nature03608.

Bago, B., Azcon-Aguilar, C., Goulet, A. and Piché, Y. 1998a. Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. *New Phytol.* **139**: 375-388.

Bago, B., Azcon-Aguilar, C. and Piché, Y. 1998b. Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. *Mycologia* **90**: 52-62.

Bécard, G. and Fortin, A. 1988. Early events of vesicular arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytol.* **108**: 211-218.

Bécard, G., Douds, D.D. and Pfeffer, P.E. 1992. Extensive *in vitro* hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. *Appl. Environ. Microbiol.* **58**: 821-825.

Calvet, C., Camprubi, A., Pérez-Hernández, A. and Lovato, P.E. 2013. *Plant growth stimulation and root colonization potential of in vivo versus in vitro arbuscular mycorrhizal inocula* : Ameri. Soc. Hort. Sci. http://hortsci.ashspublications.org/content/48/7/ 897.short.

Costa, F.A., Haddad, L.S.M., Kasuya, M.C.M., Oton, W.C., Costa, M.D. and Borges, A.C. 2013. *In vitro* culture of *Gigaspora decipiens* and *Glomus clarum* in transformed roots of carrot: the influence of temperature and pH. *Acta Sci. Agron.* **35**: 315-323. doi: 10.4025/actasciagron.v35i3.16581.

Croll, D., Corradi, N., Gamper, H.A. and Sanders, I.R. 2008. Multilocus genotyping of arbuscular mycorrhizal fungi and marker suitability for population genetics. *New Phytol.* **180**: 564- 568. doi: 10.1111/j.1469-8137.2008.02602.x

Dalpé, Y. and Declerck, S. 2002. Development of *Acaulospora rehmii* spore and hyphal swellings

under root-organ culture. *Mycologia* **94**: 850-855. doi: 10.1080/15572536.2003.11833178.

de Souza, F.A. and Declerck, S. 2003. Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. *Mycologia* **95**: 1004-1012. Doi: 10.1080/15572536.2004.1183 3016.

de Souza, F.A., Declerck, S., Smith, E. and Kowallchuk, G.A. 2005. Morphological, ontogenetic and molecular characterization of *Scutellospora reticulata* (*Glomeromycota*). *Mycol. Res.* **109**: 697-706.

Declerck, S. D'Or D., Cranenbrouck, S.L.E. and Boulenge, E. 2001. Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. *Mycorrhiza* **11**: 225-230.

Fortin, J.A., Bécard, G., Declerck, S., Dalpé, Y., St-Arnaud, M., Coughlan, A.P. and Yves, Piché. 2002. Arbuscular mycorrhiza on root-organ cultures. *Can. J. Bot.* **80**: 1-20. doi: 10.1139/b01-139.

Gerdemann, J.W. and Nicolson, T.H. 1963. Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Trans. Bri. Mycol. Soc.* **46**: 235-244.

Giovannetti, M. and Mosse, B. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrizal infection in roots. *New Phytol.* **84**: 489-500.

Ijdo, M., Cranenbouck, S. and Declerck, S. 2011. Methods for large-scale production of AM fungi: past, present and future. *Mycorrhiza* **21**: 1-16.

Karandashov, V., Kuzovkina, I., Hawkins, H.J. and George, E. 2000. Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus calendonium* in dual culture with transformed carrot roots. *Mycorrhiza* **10**: 23-28.

Kokkoris, V. and Hart, M.M. 2019. The role of *in vitro* cultivation on symbiotic trait and function variation in a single species of arbuscular mycorrhizal fungus. *Fungal Biology* **123**: 732-744. doi: 10.1016/j.funbio.2019.06.009.

Luginbuehl, L.H., Menard, G.N., Kurup, S., Van Erp, H., Radhakrishnan, G.V., Breakspear, A., Oldroyd, G.E.D. and Eastmond, P.J. 2017. Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. *Science* **356**: 1175-1178. doi: 10.1126/science.aan0081.

Maia, L.C. and Yano-Melo, A.M. 2001. Germination and germ tube growth of the arbuscular mycorrhizal fungi *Gigaspora albida* in different substrates. *Braz. J. Microbiol.* **32**: 281-285.

Mosse, B. 1959. The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. *Trans. Bri. Mycol. Soc.* **42**: 273-286.

Mosse, B. and Hepper, H. 1975. Vesicular-arbuscular mycorrhizal infections in root organ cultures. *Physiol. Plant Pathol.* **5**: 215-223. doi: 10.1016/0048-4059(75)90088-0.

Pawlowska, T.E., Douds, D.D. and Charvat, I. 1999. *In vitro* propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. *Mycol. Res.* **103**: 1549-1556. doi: 10.1017/S095375629900 8801.

Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Bri. Mycol. Soc.* **55**: 158-161.

Smith, S.E. and Read, D.J. 2008. *Mycorrhizal Symbiosis*. San Diego: Academic Press.

Tahir, D. 2003. *In vitro* culture of arbuscular mycorrhizal fungi: advances and future prospects. *Afr. J. Biotechnol.* **2** (12). 10.5897/AJB2003.000-1128.

Voets, L., De La Providencia ,I.A., Fernandez, K., Ijdo, M., Cranenbrouck, S. and Declerck, S. 2009. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. *Mycorrhiza* **19**: 347-356.